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Mutations of mitochondrial DNA polymerase gamma: an important cause of neurological disorders

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Academic dissertation
To be presented, with the permission of the Faculty of Medicine of the University of Helsinki, for public examination in lecture room 1 of the Meilahti Central Hospital of the University of Helsinki
on November 9th 2007, at 12 noon.
Helsinki 2007

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ISBN 978-952-92-2894-2
ISBN 978-952-10-4263-8
Yliopistopaino
Helsinki 2007

"Jokainen on oikeutettu mielipiteeseeni"

Tämän kirjan tarkoitus on osallistua sotaan apatiaa ja yksinäisyyttä vastaan.
Antoisia lukuhetkiä.

Abstract

The central themes of this thesis are mutations of the *POLG1* gene encoding the catalytic subunit of mitochondrial DNA polymerase gamma protein, polγ-α, their association with the wide spectrum of clinical phenotypes and tissue specificity and the biochemical characterization of particular defective mutant variants in the protein's spacer region.

PolG-holoenzyme is the sole DNA polymerase found in mitochondria. It is involved in replication and repair of the mitochondrial genome, mtDNA. Holoenzyme also includes the accessory subunit polγ-β, which is required for the processivity of polγ-α. Defective polγ-α causes accumulation of secondary mutations on mtDNA, which leads to a defective oxidative phosphorylation system. The clinical consequences of such mutations are variable, affecting nervous system, skeletal muscles, liver and other post-mitotic tissues.

In 2001, three PEO (Progressive External Ophthalmoplegia) -families were reported to carry either recessive or dominant mutations in the *POLG1* gene. Shortly thereafter, an Italian group found novel *POLG1* mutations in several Italian PEO-families. These findings prompted us to study the role of *POLG1* in patients with putative mitochondrial diseases.

The aims of the studies were:

1) Determination of the role of *POLG1* mutations in neurological syndromes with features of mitochondrial dysfunction and an unknown molecular cause.
2) Development and set up of diagnostic tests for routine clinical purposes.
3) Biochemical characterization of the functional consequences of the identified polγ-α variants.

Clinical, molecular genetic and biochemical studies were conducted. Clinical examinations consisted of detailed neurological assessments including MRI and PET scans. Most of the genetic studies were done with PCR-based methods,

including DNA sequencing, solid-phase minisequencing and denaturing high-pressure liquid chromatography (dHPLC). Biochemical characterization was accomplished with the aim of baculovirus-based recombinant protein expression and subsequent purification. *In vitro* assays included polymerase activity, DNA binding, and processivity measurements.

Studies describe new neurological phenotypes in addition to PEO caused by *POLG1* mutations, including parkinsonism, premature amenorrhea, ataxia and Parkinson's disease (PD). *POLG1* mutations and polymorphisms are both common and potential genetic risk factors at least among the Finnish population. The major findings and applications reported here are:

1) *POLG1* mutations cause parkinsonism and premature menopause in PEO families in either a recessive or a dominant manner. (Study I)
2) The common recessive *POLG1* mutations (A467T and W748S) in the homozygous state causes severe adult or juvenile-onset spinocerebellar ataxia without muscular symptoms or histological or mtDNA abnormalities in muscles. (Study III)
3) A common recessive pathogenic change A467T can also cause a mild dominant disease in heterozygote carriers. (Study IV)
4) The A467T variant shows reduced polymerase activity due to defective template binding. (Study IV)
5) Rare polyglutamine tract length variants of *POLG1* are significantly enriched in Finnish idiopathic Parkinson's disease patients. (Study II)
6) Dominant mutations are clearly restricted to the highly conserved polymerase domain motifs, whereas recessive ones are more evenly distributed along the polγ-α protein. (Studies I,III,IV)

The present results highlight and confirm the new role of mitochondria in parkinsonism/Parkinson's disease and describe a new mitochondrial ataxia. Based on these results, a *POLG1* diagnostic routine has been set up in Helsinki University Central Hospital (HUSLAB).

Contents

List of original publications

This thesis is based on the following publications:

I **Luoma P**, Melberg A, Rinne JO, Kaukonen JA, Nupponen NN, Chalmers RM, Oldfors A, Rautakorpi I, Peltonen L, Majamaa K, Somer H, Suomalainen A. Parkinsonism, premature menopause, and mitochondrial DNA polymerase gamma mutations: clinical and molecular genetic study. Lancet 2004 Sep 4-10; 364(9437):875-82.

II **Luoma PT**, Eerola J, Ahola S., Hakonen AH., Hellström O, Kivistö KT, Tienari PJ and Suomalainen A. Mitochondrial DNA polymerase gamma variants in idiopathic sporadic Parkinson's disease. Neurology 2007, in press.

III Van Goethem G, **Luoma P**, Rantamaki M, Al Memar A, Kaakkola S, Hackman P, Krahe R, Lofgren A, Martin JJ, De Jonghe P, Suomalainen A, Udd B, Van Broeckhoven C. POLG mutations in neurodegenerative disorders with ataxia but no muscle involvement. Neurology 2004 Oct 12; 63(7):1251-7.

IV **Luoma PT**, Luo N, Loscher WN, Farr CL, Horvath R, Wanschitz J, Kiechl S, Kaguni LS, Suomalainen A. Functional defects due to spacer-region mutations of human mitochondrial DNA polymerase in a family with an ataxia-myopathy syndrome. Hum Mol Genet 2005 Jul 15;14(14):1907-20.

The publications are referred to in the text by their roman numerals.

Abbreviations

ANT1	adenine nucleotide translocator
AP	apurinic/apyrimidinic
BER	base excision repair
C10ORF2	gene encoding TWINKLE DNA helicase
dNTP	deoxynucleotide triphosphate
dRP	5' terminal deoxyribose phosphate
HSP	heavy strand promoter
LSP	light strand promoter
MIRAS	mitochondrial recessive ataxia syndrome
MMR	mismatch repair
MNGIE	mitochondrial neurogastrointestinal encelophalomyopathy
MRI	magnetic resonance imaging
mtDNA	mitochondrial genome
mtSSBP	mitochondrial single-stranded DNA-binding protein
mtTFA	TFB1M, TFB2M mitochondrial transcription factors
NEM	N-ethylmaleimide
NER	nucleotide excision repair
NRF-1	nuclear respiratory factor 1
NRF-2	nuclear respiratory factor 2
NRTI	nucleoside reverse transcriptase inhibitor
NTH	thymine glycol glycosylase
O_H	origin of heavy strand mtDNA replication
O_L	origin of light strand mtDNA replication
ori	origin of replication
OXPHOS	oxidative phosphorylation
PD	Parkinson's disease
PEO	progressive external ophthalmoplegia
PET	positron emission tomography
polγ-α	DNA polymerase gamma, catalytic subunit
polγ-β	DNA polymerase gamma, accessory subunit
polG	DNA polymerase gamma, holoenzyme
POLG1	polymerase gamma gene, catalytic subunit
POLG2	polymerase gamma gene, accessory subunit
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal RNA
SANDO	sensory ataxic neuropathy, dysarthria and ophthalmoparesis
SNP	single-nucleotide polymorphism
TP	thymidine phosphorylase
tRNA	transfer RNA
TWINKLE	mitochondrial DNA helicase

Introduction

All human cells except red blood cells contain mitochondria. Mitochondria are essential double-membrane cellular organelles that “live, divide and fuse” semi-autonomously in an endosymbiotic relationship inside the cell. They contain their own genome (mtDNA), encoding 13 proteins essential for their ATP production function as well as 22 tRNAs and two ribosomal RNAs, which form part of the organelle’s internal protein synthesis machinery. Human mtDNA is a ~16.6 kb long intronless circular molecule, and its genetic code diverges from the universal code.

Mitochondria contain more than 1000 proteins encoded by nuclear genes. These are necessary to maintain the vital functions of both the organelle itself and the whole cell, including energy conversion, biosynthesis of various molecules (heme, nucleotides, amino acids and cholesterol), control of apoptosis, calcium buffering and signalling. These proteins are expressed and synthesised outside mitochondria and then imported from cytosol into the organelle through protein transport complexes embedded in the mitochondrial membranes. Interestingly, many of these nuclear-encoded proteins resemble their prokaryotic counterparts (bacteria and bacterial viruses = bacteriophages) suggesting a prokaryotic origin in addition to the bacterium-like structural features of mitochondria.

Currently, more than a hundred primary mtDNA mutations are known to cause human diseases. Moreover, a growing number of nuclear gene defects are known to cause mitochondrial disorders. The diseases known to date to be caused by nuclear gene defects affecting mitochondria may manifest as symptoms involving the central and peripheral nervous systems as well as the sensory organs, liver, heart or skeletal muscle.

One of the key players in mtDNA replication and maintenance is the mitochondrial DNA polymerase gamma, polG holoenzyme. Mutations in the mitochondrial DNA polymerase gamma catalytic subunit gene (*POLG1*) have proven to be a common and important underlying cause of many severe neurological disorders with surprisingly diverse clinical manifestations.

Review of the literature

1. Mitochondria

1.1 Origin of mitochondria

Mitochondria are thought to have been derived from endosymbiotic prokaryotes about 1.5 billion years ago as a consequence of symbiosis of oxidative bacteria and glycolytic proto-eukaryotic cells.[1] Most likely, the common ancestor was related to α-proteobacteria, a group of intracellular parasites[2]. Studies on prokaryotic and diverse mitochondrial genomes suggest that the bacterial genome that resembles mtDNA most closely is that of the intracellular parasite *Rickettsia prowazekii* [3] which seems to be the evolutionarily closest equivalent to the common ancestor of all existing mitochondria thus far known. Further evidence comes from the protozoan *Reclinomonas Americana.*[4] This bacterium-like mitochondrial genome contains 97 genes, out of which 67 genes encode proteins also found in all sequenced mitochondrial genomes. Theoretically, it could represent one of the transitional ancestors of all known mitochondria.

1.2 Structure

The overall structure and compartmentalization of mitochondria, consisting of inner and outer phosholipid bilayers (IM and OM, respectively), are compatible with the bacterial origin of mitochondria (Figure 1). The inner membrane contains cardiolipin, an unusual phospholipid, which is a characteristic component of bacterial plasma membranes. The inner membrane is highly folded into cristae and there is experimental evidence to suggest that cristae might form separate discontiguous compartments.[5] Most likely, the purpose of this folding is to maximize the inner membrane area and the volume of the intermembrane space between two membranes.

The outer membrane has a lipid composition similar to that of the cell membrane. It contains aqueous channel-forming porins, which enable diffusion of ions and small molecules (<5000-6000 daltons) through the membrane. OM and IM also contain specific transport protein complexes that control nuclear encoded protein import (TIM/TOM) and small molecule traffic permeases.[6]

The inner membrane has a higher protein to phospholipid ratio than the outer membrane, and it is practically impermeable. Only molecules such as molecular oxygen and water can diffuse freely through IM. IM embeds the five protein complexes I-V responsible for respiration and ATP production in a process known as oxidative phosphorylation (OXPHOS). Furthermore, the intermembrane space has a composition of ions and small molecules similar to that of cytosol. This space serves as a dynamic container of potential energy pumped from the matrix through the OXPHOS-complexes I, II and IV into the intermembrane space in the form of protons (H^+). This proton gradient between the intermembrane space and the matrix is the ultimate driving force of ATP synthesis, being in principle analogous to a dam and a water turbine transforming potential energy to electrical energy.[7]

In the matrix, attached to the inner membrane, reside the nucleoids. These complex inner membrane associated structures contain a few copies of mitochondrial genomes (2-10) each, as well as proteins involved in the maintenance of mtDNA, gene expression and protein translation.[8,9] The matrix is also the compartment where biosynthesis (amino acids, lipids, heme and steroids) as well as catabolic reactions (Krebs cycle, β-oxidation, urea cycle) take place.[7]

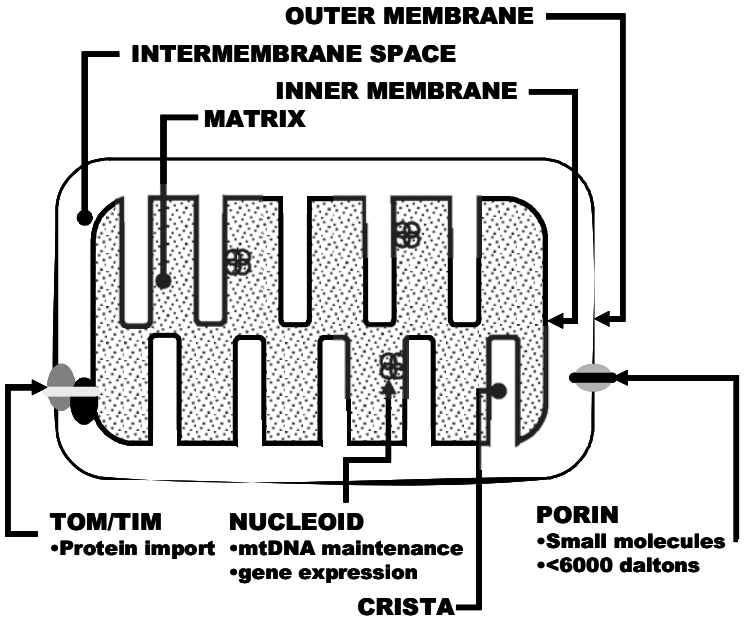

Figure 1. Schematic presentation of mitochondrial compartmentalization*.The inner membrane is highly folded (cristae), which increases its surface area and the volume of the intermembrane space. Nucleoids containing mtDNA molecules (2-10) and replication apparatus are associated with the inner membrane.*

1.3 Function

The primary function of mitochondria is to convert the chemical bond energy of fatty acids, amino acids and carbohydrates into chemical bond energy conserved as adenosine triphosphate (ATP). Mitochondria have another important role in controlling and mediating apoptosis. Additionally, mitochondria synthesize heme and steroids, regulate the cellular redox state, buffer calcium, produce heat and most likely have additional, as yet uncharacterized functions. It is therefore self-evident that mitochondrial dysfunction has the potential to cause a wide variety of diseases (reviewed by [10]).

1.3.1 Oxidative phosphorylation

The initial steps of energy conversion by oxidative phosphorylation take place in the cytosol. Pyruvate, catabolized from glucose is actively transported into the mitochondrial matrix through the outer and inner membranes. In the matrix, pyruvate dehydrogenase combines pyruvate with coenzyme A, which is fed into the citric acid cycle (Kreb's cycle, tricarboxylic acid cycle=TCA). This cycle creates three molecules of NADH and one molecule of $FADH_2$, which forward their electrons in a series of reduction/oxidation reactions within multi-subunit complexes I-IV by ubiquinone (from I and II to III) and cytochrome c (from III to IV). Gradually, step by step, electrons release their energy and finally reduce molecular oxygen into water within complex IV (Figure 2). The respiratory chain complexes I, II and IV pump protons from the matrix into the intermembrane space, creating an electrochemical gradient across the inner membrane. The proton gradient formed is exploited to drive ATP synthase (complex V), which phosphorylates ADP to ATP, hence finalizing the energy conversion process. The overall principle of this OXPHOS system is simple: the chemical bond energy of nutrients is converted into chemical bond energy of ATP and heat in a highly controlled fashion. This system employs different forms of energy such as potential (electron gradient, proton gradient) and kinetic (ATPase) in order to make this conversion occur. All of these different forms of energy originally come from the energy bound in the chemical bonds of the nutrients and the net sum of energy remains constant.[7]

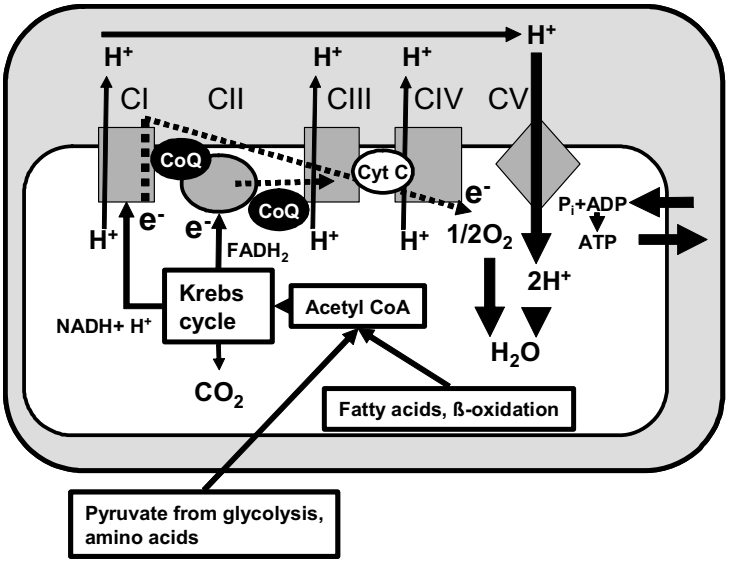

Figure 2. Principle of OXPHOS system consisting of five enzyme complexes CI-CV. *NADH and succinate are oxidized by the complexes I and II. Electrons (**e**$^-$) are transferred (dashed line) from the complexes I and II to III through the coenzyme Q (**CoQ**) and from complex III to complex IV by cytochrome c (**Cyt C**) and finally to molecular oxygen (**O_2**), which is reduced to water (**H_2O**). The electron transfer is coupled with proton (**H^+**) translocation from the matrix to the intermembrane space. It is assumed that the transport of two electrons enables the complexes I and III each to extrude four protons, while complex IV pumps two. The protons pumped into the intermembrane space flow back to the matrix through complex V providing proton gradient energy to phosphorylate adenosine triphosphate (**ATP**) from adenosine diphosphate (**ADP**) and phosphate (**P_i**).*

1.3.2 Reactive oxygen species, ROS

Since mitochondria are the site of cellular respiration, and molecular oxygen is the final acceptor for electrons carried through the respiratory chain, it is inevitable that some electrons leak from the respiratory chain to partially reduce molecular oxygen (O_2), forming superoxide anion O_2^-. This highly reactive anion is rapidly converted into hydrogen peroxide H_2O_2 by mitochondrial superoxide dismutase (MnSOD) and further into hydroxyl radical OH^- in the presence of metals such as $Fe^{2+/3+}$ (Figure 3).[11]

The role of oxygen radicals is controversial. On the one hand, they have been regarded as the obligatory and toxic side product of respiration underlying neurodegenerative diseases and ageing in general.[12-16] But there is also increasing evidence that ROS formation is strictly regulated, and that these radicals are involved in various cellular processes such as signalling and modification of the mode of action of proteins.[11,17]

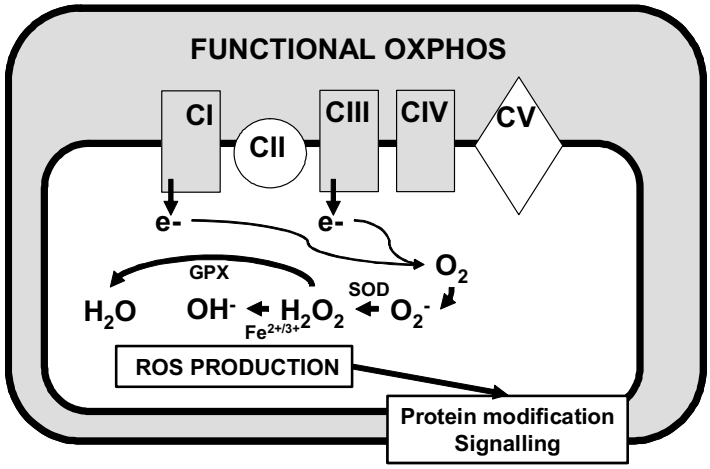

Figure 3. Reactive oxygen species, ROS. *Electrons leak mainly from complexes I and III forming superoxide from molecular oxygen. Superoxide is converted to hydrogen peroxide by superoxide dismutase (SOD). Hydrogen peroxide in turn is detoxified to water primarily by glutathione peroxidase (GPX). In the presence of transitional metal $Fe^{2+/3+}$, hydrogen peroxide is catalyzed to hydroxyl ion (Fenton reaction).*

1.3.3 Apoptosis

One of the crucial ”missions” of all proliferative cells is their ability and ”duty” to commit suicide, programmed cell death (PCD), in order to maintain cellular balance in tissues or to regulate embryonic development by removing redundant cells. One form of PCD is apoptosis, which provides defence against damaged and therefore potentially dangerous cells, which might otherwise lead to cancerous development. Apoptosis was originally described by Kerr and Wylie.[18] Apoptosis involves interplay between several proteins, and this mechanism must clearly be under rigorous control and regulation, both inhibitory and stimulatory. Up to date, the nematode *Caenorhabditis elegans* has been an important organism in studies of apoptosis (reviewed by [19]).

Apoptosis is an active process, which consists of specific and sequential morphological changes seen in dying cells, such as fragmentation of DNA, condensing of nucleus and blebbing of cytoplasm.[20] Not all forms of PCD share the characteristic shapes and sequences of apoptosis, but all types of PCD are highly regulated complex processes, which are not fully understood.

Two distinct pathways leading to apoptosis have been characterized. The extrinsic pathway is initiated by activation of cell membrane death receptors, for example Tumor Necrosis Factor (TNF) or Fas receptor.[21,22] The intrinsic pathway can be triggered by various stimuli such as DNA damage, ultraviolet radiation, chemotherapy, oxidative stress, etc. The intrinsic pathway requires disruption of mitochondrial membranes and release of the small heme protein cytochrome c into cytosol. Cytochrome c is localized in the mitochondrial intermembrane space and is an essential component of the respiratory chain carrying electrons from complex III to complex IV (cf. Figure 2). Many proapoptotic stimuli act by releasing cytochrome c into cytosol, where it can form a complex with other factors forming apoptosome passing forward the death signal.[23] Many factors that regulate the release of cytochrome c, both preventative and stimulatory, act by affecting the integrity of the mitochondrial outer membrane. This decision is affected by the relative ratio of pro- and anti-apoptotic mediators. Other mitochondria-associated factors include proteins such as Apoptosis Inducing Factor (AIF), Endonuclease G (EndoG), High-temperature requirement protein A2 (HtrA2) and Direct IAP-Binding Protein with Low pI (DIABLO)(reviewed by [24,25]).

2. mtDNA

2.1 Structure, organization and expression

Human mtDNA is a double-stranded and closed circular molecule that consists of 16569 basepairs and is about 5.2 μm long in a linearized form. Therefore, it has to be highly condensed or supercoiled to fit inside the mitochondrion as a part of the nucleoid structure. mtDNA encodes 13 essential protein subunits of the OXPHOS complexes I (7 subunits), III (1 subunit), IV (3 subunits) and V (2 subunits), two ribosomal RNAs (16S and 12S) and 22 transfer RNAs (Figures 4 and 5). The vast majority of the mitochondrial proteins are encoded by nuclear genes, including OXPHOS-subunits and proteins involved in the expression and maintenance of mtDNA, as reviewed by.[26,27] Twenty-eight mtDNA genes are encoded by heavy strand genes and nine by complementary light strand genes; all mtDNA genes lack introns.[28,29] The largest non-coding region (≈1kb) is the displacement loop (D-loop) involved in the regulation of replication and gene expression.

MtDNA is localized in the inner membrane associated nucleoprotein structures known as nucleoids (2-10 copies each, reviewed by [30,31]), which also comprise such proteins as DNA polymerase gamma catalytic α-subunit (polγ-α), DNA polymerase gamma accessory β-subunit (polγ-β), TWINKLE DNA helicase[32], single strand DNA-binding protein mtSSBP [33] and major DNA packaging protein TFAM.[34,35] Thus far, TFAM seems to be the most abundant nucleoid protein,

being present as about 1000 copies per mtDNA molecule.[36] Studies with *Xenopus laevis* oocytes have revealed putative nucleoid-associated proteins, such as adenine nucleotide translocator (ANT) and prohibitin.[37] Mammalian cells may contain thousands of copies of mitochondrial DNA organized in several hundred nucleoids.[38,39] Nucleoids form the basic units redistributing during mitochondrial fission and fusion[8] and there is evidence that they are also connected with the cytoskeleton.[38] The mtDNA molecules in nucleoids are engaged in a variety of processes, including replication and transcription. However, the detailed organization and molecular composition of the nucleoid in higher organisms is not known.

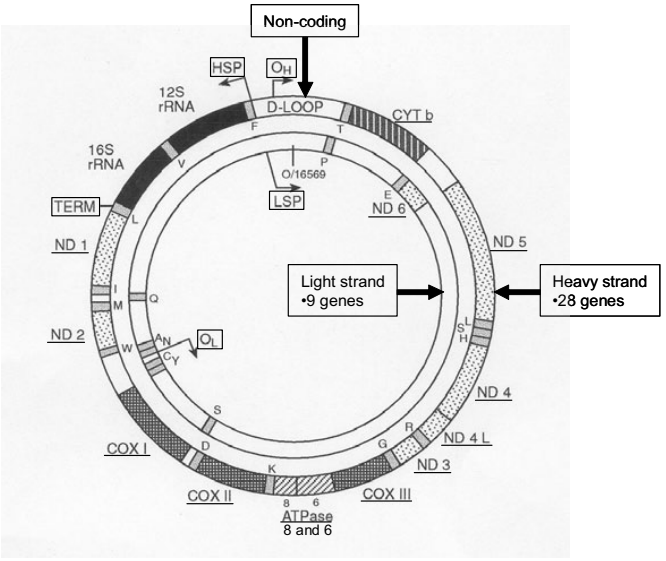

Figure 4. Organization of human mitochondrial genome. *Boxed abbreviations indicate the regulatory sites for transcription (HSP=heavy strand promoter, LSP=light strand promoter,TERM=termination site) and origins of replication (OH and OL).One letter symbols indicate the positions of tRNA genes (solid grey) and black regions of the heavy strand indicate position of ribosomal RNA genes 12S and 16S. Remaining genes encode 13 of the OXPHOS subunits (underlined). The major noncoding region is the displacement loop, i.e. D-LOOP. Modified from the thesis of Anu Suomalainen: Mutations of mitochondrial DNA in human disease (1993).*

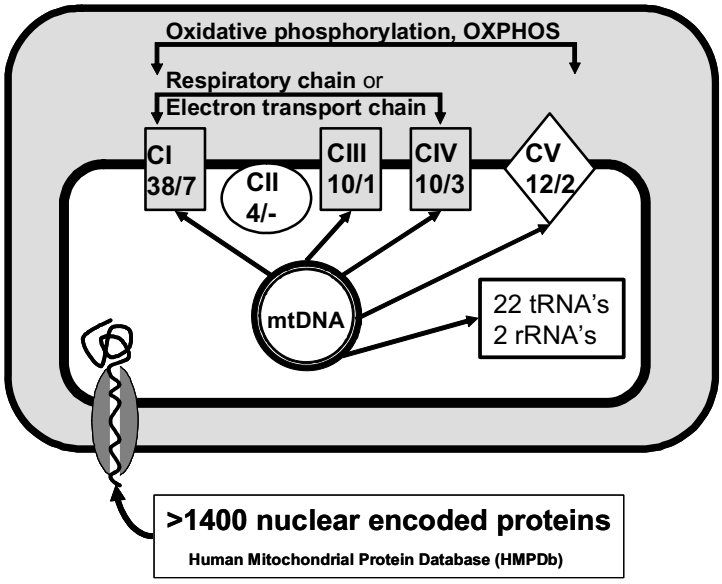

Figure 5. Only minority of the mitochondrial proteins are encoded by mtDNA. *The mitochondrial genome encodes 13 out of ~87 subunits of the oxidative phosphorylation system (OXPHOS) as well as the tRNAs and rRNAs for mitochondrial translational machinery. The subunits encoded by nuclear or mitochondrial genomes are indicated. The other mitochondrial proteins are coded by nuclear genes and imported through the membrane embedded protein complexes TIM and TOM, i.e. the translocase complexes of the inner/outer mitochondrial membranes (reviewed by[6]).*

2.2 mtDNA gene expression

Mitochondrial genes are expressed as polycistronic messenger RNA's (mRNA) from two promoters (HSP, heavy strand promoter and LSP, light strand promoter) located in the D-loop.[6,26,40] The minimal human mitochondrial transcription apparatus comprises the RNA polymerase POLRMT, the transcription factor/DNA binding protein TFAM and the two co-activator proteins TFB1 and TFB2, which also posses rRNA methyltransferase activity, although this activity is not required for transcriptional activation.[41 42 43] In addition, the transcription termination factor mTERF [44] controls the ratio of messenger RNA to ribosomal RNA. Two isoforms of the human mitochondrial transcription specificity factors TFB1 and TFB2 have been identified.[42,43] Both have been demonstrated to interact with TFAM and the mitochondrial RNA polymerase during transcription initiation. The expression of

human TFB1 and TFB2 is regulated by two nuclear respiratory factors, NRF-1 and NRF-2, as well as PGC-1 family coactivators, all of which are essential factors for mitochondrial biogenesis.[45]

Nuclear encoded protein TFAM was originally described as a mitochondrial transcription factor also involved in mtDNA maintenance.[46,47] TFAM binds conserved regulatory sequences (enhancer) within the D-loop of mtDNA and recruits other replication factors to the D-loop.[48] Because the normal TFAM cellular levels significantly exceed those needed for transcription, TFAM has been postulated to act in a histone-like fashion, providing protection to the mitochondrial genome excluding the D-loop region.[36,37,48,49]

2.3 Dysfunction leads to mitochondrial disease

In 1962 Rolf Luft described a patient with severe hypermetabolism and abnormal mitochondria in the skeletal muscle.[50] This was the first known diagnosed case of mitochondrial disease. One year later the mitochondrial genome was found.[51,52] Since then, a large number of patients have been described to have evident dysfunction of the respiratory chain. Another milestone in the history of mitochondrial disorders was the sequencing of mtDNA[28], but it took seven years before the first pathological mtDNA mutations were discovered in 1988.[53,54] Today, more than a hundred distinct mutations of mtDNA have been reported to underlie different pathological states.

Mitochondrial genetic diseases are often characterized by alterations in the mitochondrial genome, such as point mutations, deletions, rearrangements or depletion of mitochondrial DNA (mtDNA). These mutations are either primary or secondary, the latter being due to primary mutations in nuclear-encoded mitochondrial genes such as the catalytic subunit of DNA polymerase gamma (*POLG1)*, which lead to secondary mutations in mtDNA and OXPHOS dysfunction (Figures 6 and 7). The “normal” mutation rate of the mitochondrial genome is 10-20 times greater than that of nuclear DNA, and mtDNA is more prone to oxidative damage than is nuclear DNA.[55] Mutations in human mtDNA can cause premature aging and severe neuromuscular pathologies which are maternally or autosomally inherited (reviewed by [56-58]).

Mitochondrial diseases cover the disorders that are caused by mutations in either nuclear or mitochondrial genes. Mitochondrial diseases may take on unique characteristics both because of their mode of inheritance and because mitochondria are critical to cell function. However, the effects of mitochondrial disease can be quite diverse. Since the distribution of defective mtDNA may vary from organ to organ within the body, a mutation that may cause liver disease in one person might cause a brain disorder in another (Figure 8). In addition, the

severity of the defect may vary extensively. Some defects cause adult onset exercise intolerance, which is a sign of myopathy. Other defects can affect mitochondrial function more severely and cause serious multisystem consequences with early onset.

Although mitochondrial diseases vary greatly in their presentation from person to person, several major categories of the disease have been defined, based on the most common symptoms and the particular mutations that tend to cause them (see http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM)

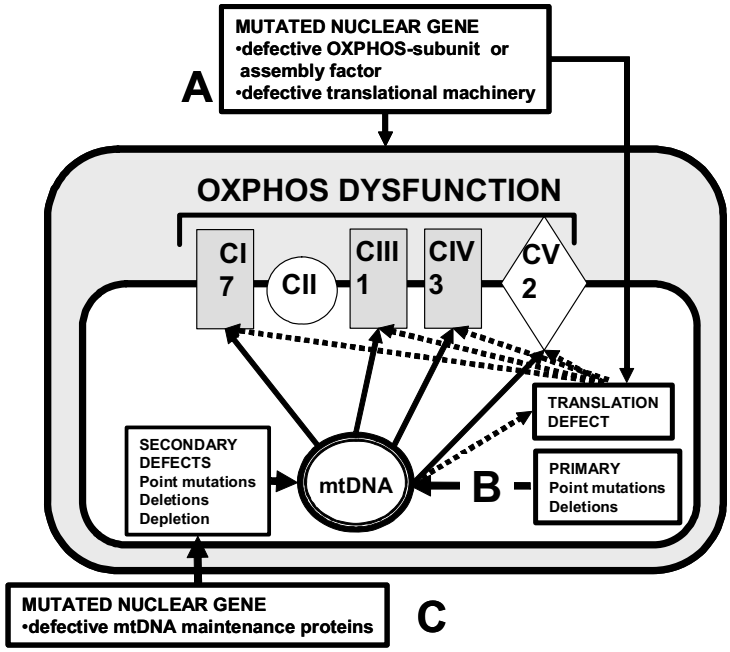

Figure 6. Three genetic pathways can lead to OXPHOS dysfunction. *A) Mutated nuclear gene encoding a defective subunit of OXPHOS complexes or an assembly factor or a defective component of mitochondrial translation machinery. B) Maternally inherited mtDNA harbours a pathogenic mutation. C) Mutation of a nuclear gene encoding defective protein involved in mtDNA maintenance leads to secondary defects in mtDNA. What B and C have in common is that the adverse effect is caused by defective mtDNA either directly by encoding abnormal OXPHOS subunits or indirectly through translational defects.*

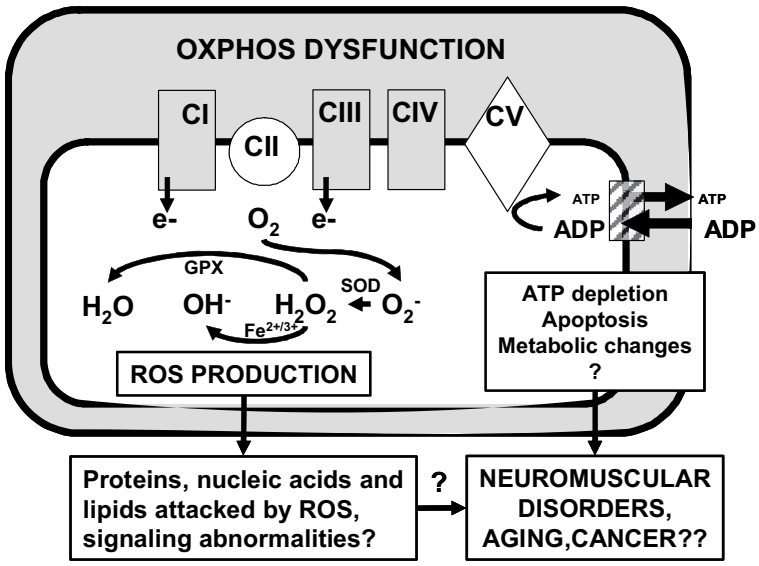

Figure 7. Dysfunction of oxidative phosphorylation leads to ATP depletion and enhanced production of reactive oxygen species (ROS). *Reactive oxygen species (ROS), or free radicals, are generated as a result of respiratory chain dysfunction (cf. Fig 3). These free radicals have at least one unpaired electron, which makes them chemically unstable and highly reactive with other molecules. Gradually, damage accumulates due to the inability of cells to handle increasing amounts of radicals.*

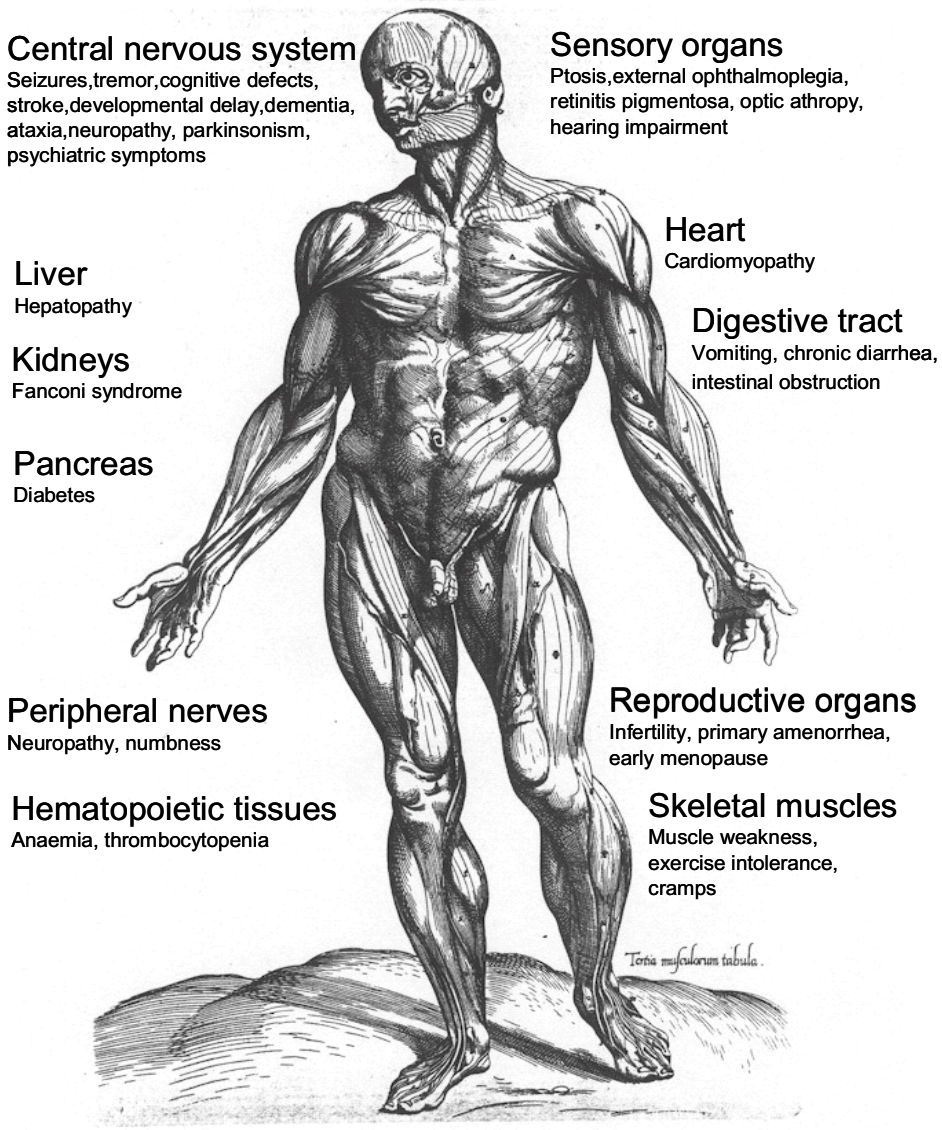

Figure 8. Mitochondrial disorders have many clinical manifestations with a wide variety of different symptom combinations (Illustration by Andreas Vesalius, 1514-1564).

2.4 Maternal inheritance of mitochondrial disease

MtDNA is inherited maternally, as compared to the nuclear gene inheritance modes, namely recessive and dominant autosomal or X-linked inheritance.[59] Nuclear genes have two copies per cell, whereas haploid germ cells have only one copy. One copy is inherited from the father and the other from the mother. Mitochondria, however, contain their own DNA and have variable numbers of copies, depending on the cell types and their energy demands. MtDNA is strictly inherited from the mother and transmitted further by the female germline. Normally, these mtDNA copies are identical, a situation called “homoplasmy”. If two different populations exist, the situation is called “heteroplasmy”.[53] The two mtDNA pools segregate into daughter cells upon cell division, and the segregation pattern determines the ratio of mutant to wild-type mtDNA in a given tissue or cells. A situation where more than two distinct mtDNA sequences occupy the mitochondria is called “pleioplasmy”. [60]

In a situation where the mother is a carrier of a heteroplasmic mtDNA pool, distinct cells and tissues may have different ratios of mutant to wild-type mtDNAs. This also applies to the ovarian precursor cells (primordial germ cells). If these precursor cells have a heteroplasmy ratio of 50/50%, for example, then the individual mature ovum can have a distinct ratio of mutant to wild-type mtDNA due to reduction of mtDNA amount and random distribution of mutant and wildtype mtDNAs after meiosis (Figure 9).[61] When the mature oocyte is fertilized, the outcome regarding disease depends partly on the initial ratio of mutant versus normal mtDNA in the individual oocyte. If there are enough cells in individual tissues or specific organs, which have reached the critical amount of mutated mtDNA, it would most likely lead to an impaired OXPHOS-system causing clinical manifestation (for a review see [62]).

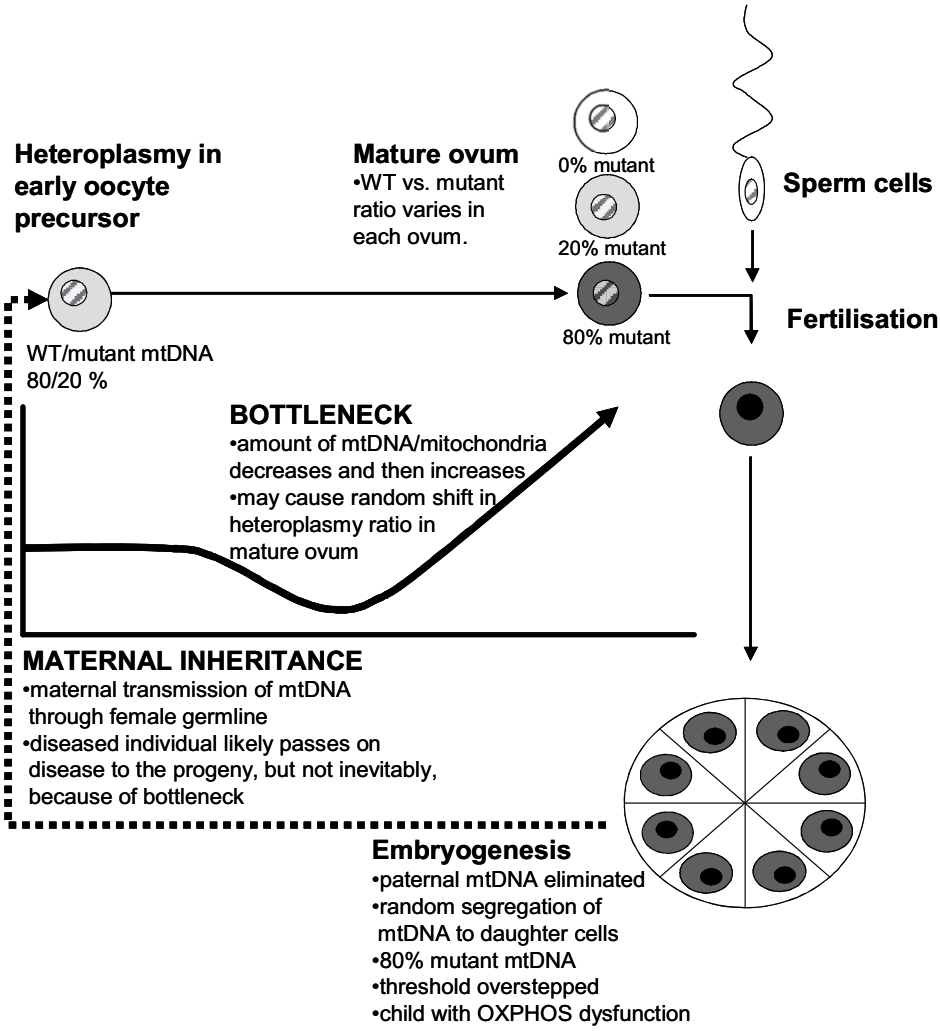

Figure 9. Concepts of maternal inheritance, mitochondrial heteroplasmy, genetic bottleneck and threshold effect. *Heteroplasmy= more than one genetically different copy of mtDNA present in cells or tissues. Bottleneck= amount of mtDNA decreases during maturation of ovum, this can change heteroplasmic ratios dramatically or even lead to homoplasmy, when there is only one mtDNA species. Threshold effect = amount of mutant mtDNA exeeds levels where OXPHOS-system does not work effectively enough.*

3. Gene expression of nuclear encoded mitochondrial proteins

The nuclear respiratory factors 1 and 2 (NRF-1/2) are transcription factors that regulate the expression of the nuclear-encoded mitochondrial proteins needed for oxidative phosphorylation, components of the mitochondrial transcription machinery and the protein import complex. The binding of NRF-1 to genomic DNA is regulated by the ATP requirements of the cell. Promoters of the human genes for the polγ-α catalytic subunit, the accessory subunit polγ-β and the mitochondrial transcription factor (mtTFA) contain consensus-binding motifs for NRF-1. NRF-2 has been shown to activate at least cytochrome oxidase subunit IV expression although it might be involved in expression of multiple respiratory genes, as reviewed by [63].

In 1999, Wu et al. showed that PGC-1 [64], a cold-inducible coactivator of nuclear receptors, stimulated mitochondrial biogenesis and respiration in muscle cells through an induction of uncoupling protein 2 (UCP-2) and through regulation of the nuclear respiratory factors (NRFs). PGC-1stimulates induction of NRF-1 and NRF-2 gene expression; in addition, they showed that PGC-1 binds to and coactivates the transcriptional function of NRF-1 on the promoter for mitochondrial transcription factor A. (reviewed by [65]).

4. mtDNA replication

The basic processes of mitochondrial DNA replication were initially elucidated with studies on budding yeast. Yeast mtDNA is ~80 kb, i.e. almost five times the size of human mtDNA, and it contains bidirectional origins of replication (ori/rep sequences numbered 1-8). This mechanism is similar to that of mtDNA replication in vertebrates and has been recently reviewed by [66].

Currently, the replication of mtDNA is not fully understood, and there are two major theories (the Clayton and Holt-Jacobs models) of how this replication is accomplished. [40,67-74] Progress has been made by identifying a growing list of proteins involved in the replication and by reconstitution of an *in vitro* mitochondrial replisome capable of synthetizing single-strand DNA, which is close to the full size of mtDNA. [75] This minimal replisome consists of polγ-α, polγ-β and TWINKLE helicase. The addition of mitochondrial single-stranded DNA-binding (mtSSB) protein enhances processivity, 5'-3' DNA helicase TWINKLE [32] unwinds the DNA, and polγ-α itself catalyses mtDNA replication.[76] *In vivo* the situtation is more complex and highly regulated, requiring additional accessory proteins and synthesis and processing of RNA,[77] and this will be discussed in more detail below.

4.1 Mechanisms of DNA replication in mitochondria

4.1.1 Clayton-model

The first model of mammalian mtDNA replication was reported in 1982.[67] According to this model, the replication of mtDNA takes place in a unidirectional and asymmetric fashion in mouse L-cells (Mouse embryo fibroblast, Moloney Sarcoma Virus transformed).[26,67,78] This model has two characteristic features. Firstly, mtDNA has two distinct origins of replication: O_H in the D-loop and O_L approximately two thirds downstream (cf. Figure 4).[79,80] Secondly, transcription initiates replication from O_H displacing the H-strand and synthesizing a new one until O_L is reached. So far, two thirds of the new H-strand has been synthesized. At this point, displacement of the H-strand exposes the O_L , from which the synthesis of the lagging strand initiates.In this model the primer for the initiation of mtDNA replication at O_H is believed to be generated by processing the transcript starting at LSP.[26,81]

4.1.2 Holt-Jacobs model

Recently, the Clayton model was challenged by a more conventional model based on studies of mtDNA replication using two-dimensional gel electrophoresis[70,82,83]. This model presumes that replication proceeds in the presence of conventional duplex replication intermediates indicating symmetric, semidiscontinuous DNA replication with coupled leading and lagging strand DNA synthesis. Initially, replication was believed to initiate at or near O_H, proceeding unidirectionally around mtDNA and suggesting coexistence of both asynchronous and strand-coupled modes of mtDNA replication.[70] Later, this model was revised by the same authors based on the assumption that mammalian mtDNA replication proceeds mainly, if not exclusively, by a strand-coupled mechanism.[82,83] In addition, replication was shown to initiate at multiple sites along mtDNA, proceeding bidirectionally. The most recent findings indicate that the mtDNA replication in verterbrates comprises elements called RITOLS (Ribonucleotide Incorporation ThroughOut the Lagging Strand). These are short stretches of ribonucleotides found in the lagging strand, indicating that there might be uncharacterized primase activity, or alternatively, these are preformed oligoRNAs hybridized to the L-strand. The initiation of the replication of the H-strand from RITOLS seems to occur in the non-coding region and is unidirectional.[74]

4.1.3 In vitro replisome

Korhonen et al.[75] provided novel insight into the biochemical aspects of mtDNA replication reconstituting a minimal mtDNA replisome. The replisome contained recombinant polγ-α and polγ-β (both catalytic and accessory subunits), TWINKLE and mtSSBP. The combination of three proteins, polγ -α, polγ-β and TWINKLE, demonstrated efficient synthesis of single-stranded DNA approximately 2000 nt in length, using double-stranded minicircle DNA as a template. Addition of mtSSB to the complex permitted synthesis of single-stranded DNA products more than 15 000 nt in length, a size similar to the mammalian mitochondrial genome.[75,76]

5. DNA polymerase gamma, polG holoenzyme

So far, altogether 16 mammalian DNA polymerases have been identified. These polymerases are essential in maintaining genetic information through faithful replication and repair. Only one of these polymerases is involved in maintaining the mitochondrial genome (mtDNA), namely DNA polymerase gamma holoenzyme (polG), which is encoded by the nuclear genes (*POLG1* and *POLG2*) and imported into mitochondria. It represents only about 2% of total cellular polymerase activity. In mammals, including humans, it has a C-terminal DNA-polymerizing activity (pol-domain) and N-terminal proofreading activity (exo-domain). These domains are separated by a spacer or linker region, which is thought to have a role in DNA binding and interaction with the processivity subunit (polγ-β) of the holoenzyme (Figure 10). Polγ-α shows some exceptional characteristics, which differentiate it from the other mammalian polymerases. These include reverse transcriptase activity, sensitivity to *N*-ethylmaleimide (NEM), capability to incorporate dideoxy-NTPs, resistance to aphidicolin and stimulation by salt.[84,85] Dysfunction of polγ-α has turned out to be an important and common cause of neurodegenerative diseases. When mutated, it causes secondary defects on mtDNA, such as deletions, point mutations and depletion, which lead to defective or reduced synthesis of mtDNA-encoded components of the respiratory chain. In addition to polG, other proteins known to be involved in mtDNA replication and maintenance are briefly reviewed.

5.1 Discovery and characterization of polG

In 1970, the first reports [86,87] describing RNA-dependent DNA polymerase activity were published. Five years later, this new polymerase was designated as polγ (polG), [88] although its cellular function was unclear.

In 1977, polG was localized to the mitochondrial compartment,[89] and evidence supporting the functional role of polG in mitochondria was obtained two years later in a study of isolated brain synaptosomes.[90] The first evidence of human mitochondrial DNA polymerase gamma was reported in 1987.[91] With the aid of homologous yeast sequences, human and fruit fly genes were cloned.[92]

The human heterotrimeric holoenzyme (195 kD) is composed of two subunits of accessory subunits (polγ-β)[84] and one subunit of catalytic polγ-α which possesses DNA polymerase,[84] 3'-5' exonuclease [93] and 5'dRP lyase activities.[94]

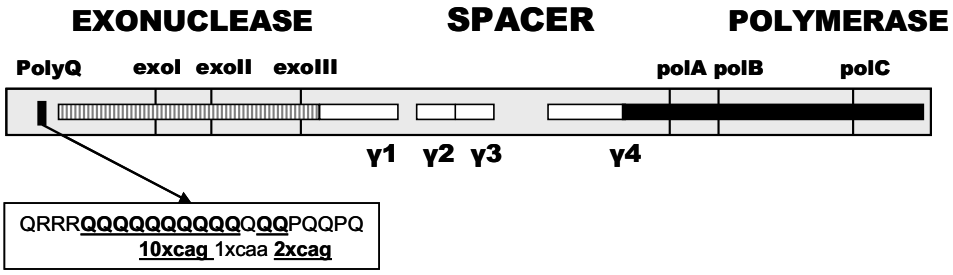

Figure 10. Linear organization of catalytic subunit of DNA polymerase gamma*. The exonuclease domain (striped horizontal bar) contains three conserved motifs (vertical black bars) as does the polymerase domain (horizontal black bar). The spacer domain consists of four moderately conserved blocks, γ1-4 (white horizontal bars) and has functions in template DNA binding, positioning and interaction with an accessory subunit. In addition, the N-terminus has short polyglutamine tract most commonly thirteen glutamine residues long. The function of the polyglutamine tract is not known. The most common polyglutamine tract is encoded by ten cag-triplets followed by one caa- and two cag-triplets. The second most common allele has eleven cag-triplets, one caa- and two cag-triplets.*

Several groups then reported an additional polypeptide that associates with the catalytic subunit. [95-98] The gene encoding this peptide was first identified in the fruit fly, and a database search revealed a partial human homolog.[99] Full-length human cDNA was cloned, expressed and purified a few years later.[93] This protein has been nominated as polγ-β, and it is the accessory subunit encoded by the nuclear gene, *POLG2*. The human accessory subunit polγ-β is required for highly processive DNA synthesis.[93,100,101] The accessory subunit forms a high-affinity, salt-stable complex with polγ-α. Reconstitution of the human complex with recombinant subunits restores salt tolerance, stimulates polymerase and exonuclease activities, and increases the processivity of the enzyme by several 100-fold. polγ-β also binds double-stranded DNA with moderate strength and

specificity. Amino acid alignment of the *Drosophila melanogaster, Xenopus laevis*, and human accessory subunits revealed significant homology to the class II aminoacyl-tRNA synthetases,[102,103] although the ATP binding and the anticodon-binding sites are impaired.[104]

Mitochondrial DNA accounts for approximately 1% of total cellular DNA, and polγ activity comprises only 1-5% of total cellular DNA polymerase activity. polγ-α belongs to the family A polymerases,[105] which includes the Klenow fragment of *E.coli*, Taq polymerase and bacteriophage T7 DNA polymerases, to mention just a few.

POLG1 is expressed and translated in cultured human cell lines that either contain or lack mitochondrial DNA, indicating that the polγ-α is stable in the absence of mitochondrial DNA *in vivo*.[92]

5.2 Molecular structure of the polγ-α catalytic subunit

So far, the three-dimensional structure of polγ-α has not been solved, although many 3-D structures of prokaryotic polymerases belonging to the same polymerase A family have been resolved (reviewed by [106]) According to these results, the predicted structure of the polγ-α polymerase domain shows well-defined fingers, thumb, and palm subdomains. This model has provided structural insights into the function of many of the conserved amino acids at the active site, which has been useful in predicting the potential effect of disease mutations. [107,108]

5.3 Molecular structure of the accessory subunit, polγ-β

A three-dimensional model of the C-terminal region of polγ-β was developed in 1999, [103] and the three-dimensional structure of the mouse accessory subunit was determined two years later. This showed that the subunit crystallized as a dimer.[104] polγ-β seems to show some structural similarities to the N-terminal domain of the subunit of the complex in *E.coli* DNA polymerase III and *E. coli* thioredoxin,[103] and the overall crystal structure is remarkably similar to that of glycyl-tRNA synthetase but the functional sites of aminoacyl-tRNA synthetases are not conserved in the polγ-β accessory subunit.[104]

Studies with deletion mutants have shown that interaction with the catalytic subunit occurs via a highly conserved C-terminal domain.[100] Furthermore, physical studies have shown that a polγ-β deletion mutant lacking the N-terminal two-helix bundle cannot dimerize.[109] In addition, the accessory subunit was shown

to bind the catalytic subunit as a homodimer forming a heterotrimeric holoenzyme. Several studies suggest that the interacting region in the catalytic subdomain is located in the early spacer domain.[110-112] Studies conducted *in vitro* show that polγ-β stimulates polymerase and exonuclease activities and increases the processivity of polγ-α in different species. [93,101,102,113]

5.4 Enzymatic activities of polγ-α

5.4.1 Polymerase activity

In addition to natural templates, human polγ-α can utilize a wide variety of DNA substrates, including activated DNA, singly primed M13 DNA and several homopolymers such as poly(dA)•oligo(dT) and poly(dC)•oligo(dG). polγ-α also possesses reverse transcriptase activity, which permits differentiation of this activity from most other cellular DNA polymerases by assay on poly(rA)•oligo(dT).[84,114] In addition to DNA polymerizing activity, copurification of 3'-5'exonuclease activity has been shown in various species.[95,97,98,115-119]

The catalytic rate of reverse transcription is higher for polγ-α than for HIV-1 reverse transcriptase, and there is also evidence of proofreading activity with the RNA template. This variety of templates might indicate that polγ-α as the sole mitochondrial DNA polymerase has additional and unknown roles in nucleic acid processing. polγ-α is active between pH values of 7.5 and 9.5, and the enzyme also requires a divalent metal cation. Mn^{2+}cations are preferred on DNA templates, whereas Mn^{2+} are required for efficient utilization of ribopolymeric templates.[84 120]

5.4.2 3'- 5' Exonuclease Activity

polγ-α-associated exonuclease activity was first characterized in several species.[97,116-118] Using a yeast homolog of polγ-α, MIP1, three exonuclease motifs were identified[105] as having significant homology with other family A polymerase exonuclease motifs. Mutagenization of the yeast exo-motifs 1, 2 and 3 retained the polymerase activity, but the strains exhibited a several 100-fold increase in the frequency of mutations of mtDNA relative to the wild-type enzyme with its intact, mismatch-specific 3'-5' exonuclease activity.[121] Subsequently, the intrinsic 3'-5' exonuclease activity of polγ-α was also shown in the human catalytic subunit and reconstituted forms of the human holoenzyme.[84,93,101,122] Exonuclease activity also has a broad pH optimum, requires a divalent metal cation and is stimulated by moderate to high concentrations of NaCl. The exonuclease shows preference for

3'-terminal mispairs in double-stranded DNA and degrades efficiently single-stranded DNA. polγ-α has a high mismatch specificity ranging from 5- to 34-fold, depending on the DNA substrate and the specific nucleotides that make up the mispair.[84,85,117,118,123] polγ-α can replicate DNA accurately with error frequencies of less than $3.8x10^{-6}$/nt, [97,116] indicating that the exonuclease contributes to replication fidelity. All family A DNA polymerases with intrinsic exonuclease activity contain conserved residues in their exonuclease active sites, which coordinate the two metal ions involved in catalysis.[124] Substitution of these residues in human polγ-α (D198A and Q200A) with alanine in the ExoI motif eliminates the detectable 3'-5' exonuclease function *in vitro.* [84] Comparison of the base substitution errors of mutant (exo^-) and wild-type polγ-α shows ~20-fold higher fidelity in the wild-type.[125]

Expression studies with human cultured cells show accumulation of point mutations when exo-deficient polγ-α is introduced to cells.[126] Several mutations associated with PEO or Alpers syndrome have been found in the exonuclease domain, but none of them are located within the conserved exo-motifs.[127-138] All these mutations are found as compound heterozygotes and mostly inherited as a recessive trait.

The catalytic subunit itself has high base substitution fidelity resulting from high nucleotide selectivity and exonucleolytic proofreading.[125] However, when copying homopolymeric sequences longer than four nucleotides, polγ-α has lower frameshift fidelity, suggesting that homopolymeric runs in mtDNA may be especially sensitive to frameshift mutations.

5.4.3 5' dRP-lyase activity of polγ-α

dRP-lyase activity, which catalyses the cleavage of the 5'-terminal dRP sugar residue from apurinic/apyrimidinic sites, was first found in *Xenopus laevis.*[139,140] The authors also demonstrated that the dRP-lyase reaction proceeds via the formation of a covalent enzyme•DNA complex that is converted into an enzyme•dRP intermediate. In base excision repair pathways, the damaged base is recognized and removed by DNA glycosylase and AP endonuclease activities. dRP lyase activity allows polγ-α to remove the dRP residue and to fill the resulting single-nucleotide gap, thereby generating a substrate for DNA ligase.[94] The accessory subunit can increase the efficiency of this reaction by increasing the lyase activity and the ability of the enzyme to locate the damage site on DNA, presumably by enhancing DNA binding.[141]

5.4.4 polG in mitochondrial DNA repair

In addition to damage by reactive oxygen species, mtDNA is also damaged by exposure to radiation and chemicals. Repair of the damage to mtDNA is necessary to avoid the accumulation of point mutations and/or deletions. Certain types of DNA damage can be efficiently repaired in mitochondria. As the only DNA polymerase present in mitochondria, polG is involved in these repair processes such as base excision repair (BER).[94,142,143]

5.5 Proteins with putative interactions with polG

5.5.1 TWINKLE, mitochondrial 5'-3' DNA helicase

In 2001, Spelbrink et al. [32] reported a nuclear gene (*PEO1*), which encodes a mitochondrial DNA helicase TWINKLE. The protein is similar to bacteriophage T7 gp4 protein, which has N-terminal primase activity and C-terminal DNA helicase activity and is required at phage replication fork.[32] The authors reported also four ethnically distinct autosomal dominant PEO-families carrying mutations in this gene. The authors found that *C. elegans* and *Drosophila* have similar proteins, also related to T7 gp4. Although homologous to T7 primase-helicase, TWINKLE has not been shown to harbour primase activity. Instead, it has been shown to localize in mitochondrial nucleoids and cells overexpressing twinkle had an increased mtDNA helicase activity. [8]

TWINKLE itself has been shown to unwind only short stretches (<20 bp) of dsDNA in the 5'-3' direction.[144] polG holoenzyme itself is unable to use dsDNA as a template, however, when the polG and TWINKLE are combined, they form a processive replication machinery, a replisome, which can utilize dsDNA as template to synthesize ssDNA molecules of about 2 kb. Addition of the mitochondrial single-stranded DNA-binding protein (mtSSBP) stimulates the reaction, generating DNA products of about 16 kb, the size of the mammalian mtDNA molecule.[75]

Polγ-α and its dsDNA-binding activity are required for the function of the mitochondrial DNA replisome. The dsDNA-binding activity of accessory subunit is not required to stimulate the DNA synthesis rate or the processivity of the polγ-α but is needed for functional interactions between the polymerase gamma holoenzyme and TWINKLE. There is no proof for physical interactions between the components of the mtDNA replisome, but functional interaction may explain the dependence on TWINKLE and the polG holoenzyme for DNA synthesis.[76]

5.5.2 Single strand binding protein, mtSSBP

In 1995, Tiranti et al.[145] reported that the gene encoding mitochondrial single-stranded DNA-binding protein (mtSSBP) is a housekeeping gene involved in mitochondrial biogenesis. mtSSBP is a small protein (132-amino acids) that acts as a homotetramer to stabilize the displaced single strand DNA during mtDNA replication. The most likely role of mtSSBP is the prevention of the formation of secondary ssDNA structures, which could stop the polG, causing replication stalling.

Farr et al. have shown that functional interaction of mtSSBP and polG enhance the overall activity of polG in *Drosophila* embryos by increasing primer recognition and stimulating the initiation of DNA strand elongation.[146] Later they showed that DNA-binding mutants of mtSSBP are defective in stimulation of DNA synthesis by polG. Knock-down of mtSSBP reduced the expression level of the gene to less than 5%, resulting in mtDNA depletion and growth defects in Schneider cells. Overexpression of mtSSBP restored the cell growth and mtDNA copy number. [147] Korhonen et al.[75] have also shown the stimulatory effect of mtSSBP to mtDNA replication with human orthologs *in vitro,* as mentioned earlier.

6. Human disorders associated with defective polγ-α

POLG1, is one of the nuclear genes that is associated with mitochondrial DNA depletion or deletion disorders when mutated. Dysfunction of mitochondrial DNA polymerase gamma has been associated with disorders such as progressive external ophthalmoplegia (PEO),[138] Alpers syndrome,[148] mitochondrial neurogastrointestinal encephalomyopathy (MNGIE)[149] or sensory ataxic neuropathy, dysarthria and ophthalmoparesis (SANDO).[132] Also, polyglutamine tract variants in the N-terminus of polγ-α have been implicated in male infertility in some instances, [150] although several reports have failed to replicate that association.[151,152] Since 2001 there are several publications reporting over 80 distinct *POLG1* mutations associated with diverse clinical manifestations (Figure 11). [111,127-134,136,138,148,149,153-173]

6.1 Progressive external ophthalmoplegia, PEO

In 1989 multiple large-scale deletions of mtDNA isolated from muscle biopsies were demonstrated in Italian families with the heritable autosomal dominant form

of PEO (adPEO) by Zeviani and colleagues.[174] PEO usually has late onset (between 18 and 40 years of age) with bilateral ptosis and progressive weakening of the external eye muscles, resulting in blepharoptosis and ophthalmoparesis, proximal muscle weakness, wasting and exercise intolerance. Frequent additional features include cataracts, hypogonadism, dysphagia and hearing loss.[175-177] The skeletal muscles of PEO patients contained ragged-red fibers and showed impaired activity of respiratory chain enzymes. In 1992 Suomalainen et al.[178] reported an adPEO patient with a multisystemic disorder in which one of the major clinical symptom was depression. They also found that the patient had significant amounts of deleted mtDNA species in skeletal muscles, brain and heart.[178,179] Both autosomal dominant and usually more severe autosomal recessive inheritance can occur, depending on the causative mutation and what gene is affected.

Van Goethem et al.[138] identified three Belgian families with PEO and multiple mtDNA deletions. The diagnosis was based on clinical symptoms of PEO and muscle weakness, the presence of ragged-red fibers and multiple mtDNA deletions in muscle biopsies. Electron microscopy showed subsarcolemmal accumulation of abnormally structured mitochondria with paracrystalline inclusions. The inheritance pattern in one family was autosomal dominant, whereas the other two families likely had autosomal recessive inheritance. In 2001, the first report was published describing the same families in which PEO cosegregated with *POLG1* mutations.[138] In addition to *POLG1* three genes have been associated with chronic PEO, namely *ANT1*[180], *PEO1*[32] and *POLG2*[181]

6.2 Alpers syndrome

Bernard Alpers described in 1931the neuropathology and clinical features of a 4-month-old girl with a one month illness characterized by intractable generalized seizures.[182] He termed the disorder 'diffuse progressive degeneration of the gray matter of the cerebrum'. Alpers syndrome is usually characterized by psychomotor retardation, intractable epilepsy and liver failure in infants and young children. Definitive diagnosis is confirmed by postmortem examination, showing typical morphological changes of the brain and liver, such as spongiosis, neuronal loss, and astrocytosis, which progresses down through the cortical layers, hepatocyte loss, bile duct proliferation, fibrosis, and often cirrhosis.[183] The illness usually begins in early life with convulsions. A progressive neurologic disorder characterized by spasticity, myoclonus and cognitive decline ensues. Status epilepticus is often the terminal development.

In 2004, the first report of *POLG1* mutations underlying Alpers syndrome was published.[148] The authors reported an Alpers patient with reduced electron

transport chain function, dicarboxylic aciduria, fulminant hepatic failure, refractory epilepsy and lactic acidosis which resulted in death at 42 months. Sequencing revealed that the patient was compound heterozygous, predicting Q873X and A467T amino acid substitutions.[167] Since then, reports have been published of several other mutations found in *POLG1* in 20 independent pedigrees.[129,134,156,164,168,184] At least 16 mutations associate with Alpers syndrome. In all cases, the *POLG1* mutations found in Alpers patients are recessive. Many of these mutations also exist in PEO with recessive mode of inheritance.

6.3 Sensory ataxic neuropathy, dysarthria and ophthalmoparesis, SANDO

SANDO is a clinically heterogeneous systemic disorder with variable features resulting from mitochondrial dysfunction. It shares clinical characteristics with autosomal recessive progressive external ophthalmoplegia and mitochondrial neurogastrointestinal encephalopathy syndrome.[185]

Van Goethem et al. (2003) reported a man with SANDO having disturbance of balance, which progressed slowly during the third decade and became disabling with frequent falls.[132] At the age of 39, he presented with moderately severe external ophthalmoparesis, mild dysarthria and ataxic gait without other muscle weakness. The patient showed thalamic lesions on neuroimaging. The patient's parents, both 70 years of age, were clinically normal, as was his only brother. Sequence analysis of patients *POLG1* revealed two pathogenic mutations predicting amino acid substitutions A467T and R627W.

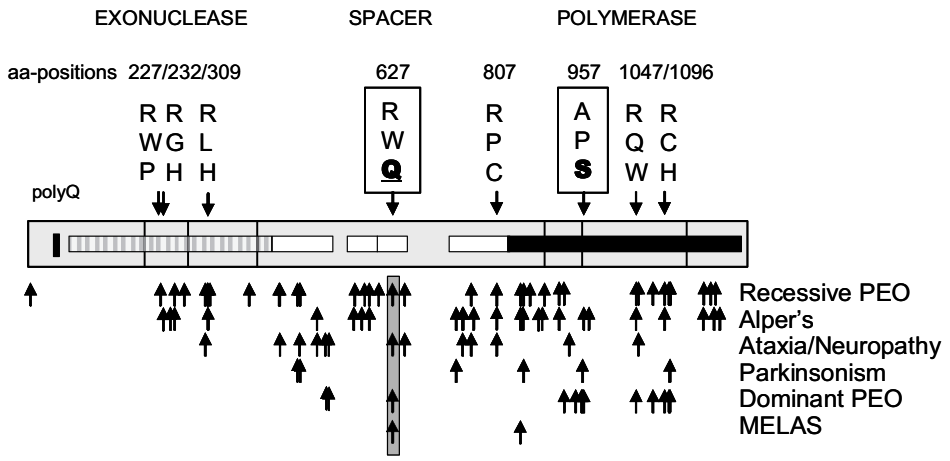

***Figure 11*. Positions of polγ-α substitutions along the primary sequence of protein.** *Arrows below the linear structure of polγ-α indicate the positions of missense substitutions associated with different disorders. The arrows above indicate the positions of the amino acids, which have been reported to harbour two different substitutions: R227W or P, R232G or H, R309L or H, R627W or Q, R807P or C, A957P or S, R1047Q or W and R1096C or H. The bold amino acid symbols indicate dominant mutations.The R627Q substitution has been also found in recessive MELAS. Dominant mutations are mainly clustered within the polymerase domain (black horizontal bar) and recessive ataxias always have at least one mutation within the spacer area. Grey box indicates the R627Q or R627W substitution, which has been shown to act in recessive (MELAS/Ataxia)) and dominant (Ataxia) manner (see http://dir-apps.niehs.nih.gov/polg/, and references therein). Striped horizontal bar indicates the location of exo-domain.*

7. Mouse models of exonuclease or polymerase defective polγ-α

Thus far there have been reports of mouse models expressing defective polγ-α either ubiquitously or in targeted manner. Both homo- and heterozygous mice have been used in these studies.

Substitution of a highly conserved aspartate residue with alanine in the second exonuclease motif (D257A) produced homozygous knock-in mice expressing a exonuclease-deficient form of the catalytic subunit of polγ-α.[186] These knock-in mutator mice developed a mtDNA mutator phenotype with up to a 5-fold increase in the number of point mutations as well as a significant increase in mtDNA

deletions. The increase in somatic mtDNA mutations was associated with a reduced life span and premature aging, indicating that an increase in mtDNA mutations contributes to the aging process.

Study of a similar exonuclease-deficient knock-in mouse showed that the increase in mtDNA mutations induces apoptosis but is not associated with increased production of reactive oxygen species.[187] With more sensitive methods Vermulst et al. [188] claimed that the mutation frequency in mutator mouse mitochondria was more than ten times lower than was reported earlier. They detected an 11-fold increase in mitochondrial point mutations with age and that the mutator mice were able to sustain a 500-fold higher mutation burden than normal mice, without any obvious features of rapidly accelerated aging, they suggested that mitochondrial mutations do not limit the lifespan of wild-type mice.[188] Yamasoba et al. 2006 [189] studied the role of accumulation of mtDNA mutations in the development of age-related hearing loss using heterozygous mutator mouse, which showed accelerated aging primarily due to increased apoptosis. Mice had moderate hearing loss and degeneration of the hair cells, spiral ganglion cells and stria vascularis by 9 month of age, while wild-type animals did not. They claimed that decreased energy metabolism due to accumulation of mtDNA mutations/deletions and decline of respiratory chain function play an important role in the manifestation of AHL.

In 2007, Lewis et al.[190] expressed mutated human *POLG1* in murine heart. The mutation predicts amino acid substitution from tyrosine to cysteine at the position 955 (Y955C) and it is the most common mutation found in adPEO. In this study cardiac targeting of Y955C-polγ-α caused cardiomyopathy, mitochondrial oxidative stress, premature death and mtDNA depletion in the hearts of heterozygous mice.

Accumulation of mtDNA deletions and mitochondrial dysfunction in the brain has been hypothesized to cause bipolar disorders, since many PEO-patients have similar problems.[191] Heterozygous mice carrying a mutation in the first exonuclease motif of polγ-α (D181A) showed altered diurnal activity rhythm and periodic activity change associated with the estrous cycle. The phenotype was worsened by administration of a tricyclic antidepressant, but improved after lithium treatment.[192]

8. Biochemical characterization of pathogenic *POLG1* polymerase domain substitutions

Most of the dominant *POLG1* mutations responsible for developing PEO-disease have been mapped to the polymerase domain of polγ-α.[193] Graziewicz et al. 2004 studied four pathogenic amino acid substitutions, G923D, R943H, Y955C, and A957S, which were previously shown to associate with the dominant form of PEO.[108] Exonuclease deficient double mutant of polγ-α with these substitutions were characterized biochemically. Two of the substitutions, R943H and Y955C, change side chains that interact with the incoming dNTPs. Proteins carrying these substitutions retain less than 1% of the wild-type polymerase activity and display a severe decrease in processivity. The significant stalling of DNA synthesis and the low catalytic activities of both enzymes are the two most likely causes of the severe clinical presentation in R943H and Y955C heterozygotes. The substitution of Y955 to cysteine also increases nucleotide misinsertion replication errors 10- to 100-fold in the absence of exonucleolytic proofreading.[194] The G923D and A957S recombinant forms of polγ-α showed less than 30% of the "wild-type" (exo^-) polymerase activity. This is consistent with the milder clinical manifestations of PEO patients carrying these mutations. Because both copies of *POLG1* are expressed, autosomal dominant mutations are thought to produce proteins that compete with the wild-type polγ-α in a dominant negative manner. In addition a slight increase in DNA-binding efficiency was observed in three of the four polγ-α variants: G923D, R943H and A957S.

Aims of the study

In 2001, Van Goethem et al.[138] reported three PEO families carrying either recessive or dominant mutations in the *POLG1* gene. Shortly after that, Lamantea et al.[128,130] found novel *POLG1* mutations in several Italian PEO families. At that time already, it was obvious that mutations in the *POLG1* gene could cause either recessive or dominant diseases with interfamilial phenotype variability. This prompted us to characterize the role of *POLG1* in different neurological diseases. The specific aims of the study were as follows:

1. To study the role of *POLG1* mutations in patients with known mitochondrial disorders.
2. To identify new disease entities caused by *POLG1* mutations.
3. To develop and set up diagnostic *POLG1* routine analysis for clinical purposes.
4. To study the biochemical consequences of disease mutations *in vitro*.

Materials and methods

1. Patients, control individuals, cell cultures, biopsies and autopsies.

All patient samples were taken with oral or written informed consent. The studies were approved by ethical committees of Helsinki University Central Hospital and collaboratory institutes. The study material included:

Study I: Seven families (two Finnish, one British and four Swedish) with progressive external ophthalmoplegia, parkinsonism and premature menopause with a total of 22 patients.

Study II: 140 idiopathic Parkinson's disease patients and their 127 spouses as controls. In addition, the *POLG1* polyglutamine tract of 585 healthy controls and 127 patients with other neuromuscular disorders were analysed.

Study III: Five European ataxia families (one British, two Finnish and two Belgian) with alltogether eight patients. In addition, 168 Belgian and 70 Finnish controls were analysed

Study IV: One Austrian family with PEO/ataxia and neuropathy. Alltogether ten family members were analysed.

Primary cell lines (IV)

Lymphocytes were isolated from peripheral venous blood with heparin or EDTA as anticoagulant, using LymphoprepTM Tubes (Nycomed Dharma As) according to the manufacturer's instructions and immortalized with the Epstein–Barr virus. The cultures were maintained in RPMI 1640 (Gibco BRL) and supplemented with 20% fetal bovine serum (Gibco BRL), 2 mM Glutamax (Gibco BRL) and penicillin–streptomycin, respectively (Gibco BRL) at a density of 3x106 cells/ml at 37°C in an incubator with 5% CO_2.

DNA and RNA extraction (I-IV)

Total lymphocyte, muscle and lymphoblast DNA were extracted using standard methods. Total RNA from lymphoblasts was isolated by the RNAeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Samples were treated with RNase-free DNase (Stratagene) to avoid carry-over of the DNA template into the reverse transcription and PCR amplification.

Sequencing of *POLG1* (I-IV)

All exons were amplified by PCR: 94°C for 10 min; 35 cycles of 94°C for 1 min, 60°C for 30 s and 72°C for 1 min and final extension 72°C for 10 min using 1 U of AmpliTaq Gold-polymerase (Roche). The intronic primers flanking all *POLG1* exons were according to Van Goethem et al.[138] The PCR products were checked by agarose gel electrophoresis, purified by the QIAEX II Gel Extraction Kit (Qiagen) and sequenced using the BigDye terminator Ready Reaction Kit v.3 on a 3100 Genetic Analyzer Automatic Sequencer (Applied Biosystems).

Reverse transcription and polymerase chain reactions (IV)

Titan One Tube RT–PCR System (Roche) was used to amplify (see primers below) the fragment under study. The cycling conditions were as follows: 58°C for 30 min; 94°C for 2 min; followed by 35 cycles of 94°C for 20 s, 58°C for 30 s and 68°C for 1 min and finally 68°C for 10 min. The absence of DNA carry-over was controlled by parallel reaction without initial cDNA synthesis before the amplification steps. RT–PCR was used together with mini-sequencing in order to quantitate the conceivable allele specific expression bias.

Solid-phase mini-sequencing (IV)

Initial PCR amplification was done by AmpliTaq Gold-polymerase (Roche). Solid-phase mini-sequencing was done according to standard reaction conditions. The part of exon 7 harbouring the A467T mutation was amplified using a 5'-biotinylated forward primer (5'-accagaactgggagcgttac-3') and an unmodified reverse primer (5'-ctacctctctcctgagagca-3'). Detection and quantitation of the wild-type or mutated base were done by utilizing a detection primer (5'-cagctggcaggcatcattgg-3') and extension with a tritiated deoxy [1',2',5-3H] cytidine 5'-triphosphate and deoxy [methyl-1',2'-3H] thymidine 5'-triphosphate (Amersham Biosciences), respectively. Incorporation of the nucleoside triphosphates was done by a thermostable DNA polymerase from Thermus brockianus (DynaZymeTM/II, FinnZymes).

The part of exon 23 harboring the Q1236H variant was amplified with a 5'-biotinylated forward primer (5'-ttgaggtggcatcctaacca-3') and an unmodified reverse primer (5'-acgggagcaaatacagagcc-3'). Detection of both variants was done by a detection primer (5'-cagctggcaggcatcattgg-3') with tritiated deoxy [1', 2', 5-3H] cytidine 5'-triphosphate and deoxy [3H] adenosine 5'-triphosphate (Amersham Biosciences).

mtDNA analysis (III,IV)

Long-PCR analysis was performed using DyNAzyme EXTTM polymerase (Finnzymes) according to the supplier's instructions for standard reaction conditions (0–10 kb). The primers used for this assay were located at the nt positions 8238–8262 (forward) and 16 496–16 465 (reverse), amplifying a region of mtDNA that often harbours multiple mtDNA deletions. The cycling conditions were as follows: 94°C for 2 min; 35 cycles of 94°C for 15 s, 63°C for 30s, 72°C for 2 or 6 min and final extension at 72°C for 10 min. The optimal amount of template was titrated to be 10 ng. By reducing the amount of template, we were able to amplify deletions only from patient samples and never in age-matched controls (muscle samples from healthy siblings of PEO patients). Two separate PCR reactions for each sample were set up, with otherwise similar reaction conditions but different extension times. Six minutes extension was used to visualize the 8.2 kb product from the normal mtDNA molecule, and 2 min was used to favour the amplification of the deleted molecules. The total DNA extracted from the muscle biopsy sample was used for Southern blot analysis as described in detail. The DNA was restriction-digested with BamHI or PvuII restriction enzymes (Fermentas). Both linearize mtDNA but from separate sites. The samples were electrophoresed through a 0.8% agarose gel, and complete digestion was confirmed in ethidium bromide staining. The DNA was transferred on a Hybond-N

(Amersham Biosciences) nylon membrane according to standard procedures, and the membranes were hybridized with a probe containing a cloned fragment of mtDNA (nt 1–740) and random-labelled to a specific activity of 1x108 c.p.m. / µl, in order to detect deleted mtDNA species. The same membrane was rehybridized with a nuclear probe 18S rRNA, cloned in pBR322, in order to quantitate the mtDNA signal versus genomic DNA, to detect possible mtDNA depletion. The radioactive signal was detected by scanning PhosphorImager screens (Fuji Photo Film Co.) with Typhoon 9400 Imager (Amersham Biosciences) and quantitated with the ImageQuant 5.1 software (Amersham Biosciences).

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Statistical analysis (II)

We tested the null hypothesis of independent occurrence of rare and common repeat length classes separately in PD patients, spouse controls, patient controls and healthy control with the Fisher's exact test (http://statpages.org/ctab2x2.html) The p-value given is two-tailed.

POLG1 haplotype frequency estimation was carried out using the expectation-maximization algorithm as implemented in the HAPLO and SNPHAP software (http://www.gene.cimr.cam.ac.uk /clayton/ software/snphap.txt). HAPLO was used to estimate haplotype frequencies in the PD and control groups and to calculate a global p-value for the association with PD. SNPHAP was used to analyze the association of individual haplotypes with PD. For this purpose, we selected amino acid variants or non-coding SNPs with minor allele frequencies of >5%. Four markers were included (rs223829, L752L, rs2302084, Q1236H), which were tested under 4-locus analysis with the HAPLO program (global test). Haplotype patterns were also analysed with the SNPHAP software application (individual haplotype tests).

dHPLC (I)

Automated denaturing high performance liquid chromatography instrumentation was used (Agilent Technologies, Palo Alto, CA, USA) to search for mutations in one patient (family K). We amplified exons according to a published protocol[138] with few exceptions: the initial denaturing step was 10 min with AmpliTaq Gold (Applied Biosystems), with a final concentration of 1 U/50 µL reaction. We obtained the optimum melting temperature for every amplicon with an algorithm.

Results

1. *POLG1* mutations in new clinical phenotypes (studies I-IV)

In study I we showed that PEO, parkinsonism and premature menopause cosegregated with *POLG1* mutations. We found three new *POLG1* mutations and one previously described mutation in seven pedigrees with progressive external ophthalmoplegia. This prompted us to study further the role of *POLG1* mutations in Finnish sporadic idiopathic Parkinson's disease patients. In that study (II) we showed that rare polyglutamine tract length variants associated with idiopathic sporadic PD.

In study III we found one previously described and one new *POLG1* mutation in patients who had ataxia with various combinations of other neurological symptoms. This study also showed that even patients who carry the same mutations may have very different clinical phenotypes with or without muscle abnormalities, such as ragged-red fibers or mtDNA deletions. Another PEO/ataxia family was analysed in study IV.

In this family two *POLG1* two mutations segregated, one of them *in cis* with a polymorphic coding region variant. The index patient had the most severe phenotype carrying two mutations predicting A467T and R627Q substitutions and one additional polymorphic change Q1236H *in cis* with R627Q. Both mutated alleles were shown to cause mild or moderate clinical phenotype, A467T caused a mild ptosis and R627Q/Q1236H caused early-onset ptosis and gait disturbance.

1.1 General symptoms of patients in studies I, III, IV

In study I the initial symptoms and signs of disease in the patients were ptosis, progressive external ophthalmoplegia, and, in most individuals, peripheral neuropathy, which was often painful; parkinsonism manifested several years later. Most of the women had poorly developed secondary sex characteristics (breasts, pubic hair) and they experienced primary or secondary amenorrhoea before the onset of neurological symptoms. Cataracts were generally present. The progressive external ophthalmoplegia in the patients was consistent with a muscle disease. The patients also had typical muscle morphological findings of mitochondrial myopathies such as ragged-red fibres. They responded positively to levodopa treatment and its effect was long term.

In study III we studied eight patients from five unrelated families. The patients had adult or juvenile-onset sensory ataxic neuropathy in combination with various central nervous system (CNS) abnormalities with no apparent skeletal muscle involvement. Muscle biopsy samples showed no or minor signs of mitochondrial disease in morphologic, biochemical or mtDNA analyses. Two of the eight patients died at ages 36 and 39 years without extraocular muscle involvement. In the other six patients, PEO was absent, although minor signs of PEO appeared at a late disease stage in five.

In study IV, the patients of the Austrian family had clinical manifestations from late-onset ptosis to early-onset ptosis together with gait disturbance. The index patient was most severely affected having ataxia and early-onset PEO (IV). However, long-range PCR did show only low levels of multiple mtDNA deletions in skeletal muscles of index patient (IV).

2. *POLG1* mutations in parkinsonism and in idiopathic Parkinson's disease (I, II)

2.1 *POLG1* mutations associated with PEO, parkinsonism and premature menopause (I)

In the seven families four *POLG1* mutations were identified, one of which,Y955C, was previously described.[130,138] Y955C was found in four families (L,S,C,E) and it showed a dominant mode of inheritance, as all carriers were affected (Figure 12). A new mutation in family N, R953C changed a conserved amino acid at the polymerase active site motif polB (Figure 10). Another new mutation, A1105T was present in families K and V. The second mutation in family V, N468D, was found neither in controls nor in the literature, and it changed an amino acid highly conserved in vertebrates. Co-segregation of parkinsonism with *POLG1* mutations was seen in five families (V,L,S,C,K). In families E and N, parkinsonism was not detected. MtDNA analysis by Southern blotting was done in muscle DNA of two patients, one carrying Y955C and the other having N468D *in trans* with A1105T. Both patients had clearly multiple deletions of mtDNA (I).

2.2 polγ-α variants in idiopathic Parkinson's disease (II)

Significant clustering of rare non-10/11Q variants of the *POLG1* CAG-repeat (Figure 10), encoding a polyglutamine tract was found in Finnish idiopathic PD patients (10%) as compared to their spouses (3.5%, p=0.003), population controls (4.3%, p=0.001) and patients with other neuromuscular, non-parkinsonian disorders (5.3%, p=0.05). If all control groups are pooled together the frequency of rare variants was 4.3% and Fisher's Exact Test gave a two-tailed p-value of 0.0001. To determine the frequencies of non-10/11Q alleles in other populations we pooled the data from previously published random Eurasian population samples, which resulted in a frequency of 2.6% (76 out of 2922 chromosomes) indicating that non-10/11Q alleles are relatively rare.[150,195-198]

Further, we found eight amino acid substitutions, but none of them were clearly associated with PD since most of them were also found in spouse controls. A novel missense change S1230F was found in one PD case, and this was not present in 254 spouse control chromosomes, but the patient's healthy aged sister also carried this substitution. Four other novel amino acid variants were found. Q49E substitution was found *in cis* with L392V in one PD case and two controls. P241L and L392V were found in one control each. R722H was found in two PD patients. One of them also had Q1236H and the other had a rare 12Q allele. Three previously reported common coding region polymorphisms (Q1236H, E1143G, K317K) were found in similar frequencies in PD patients and spouse controls (12.5 vs. 16.5%, 1.8 vs. 2.4% and 1.1 vs. 1.2%, respectively, all p-values >0.2). A previously reported variant Y831C [166] was found in combination with Q1236H in one PD patient and as a single substitution in five controls.

Four locus analyses, utilizing the markers rs223829, L752L, rs2302084 and Q1236H, failed to show evidence for a specific *POLG1* haplotype association with PD (global p>0.1; individual haplotype p-values >0.08). One individual haplotype (9Q, rs223829=C, L752=C, rs2302084=A and Q1236=G) out of three had almost 4-fold frequency among PD patients vs. spouse controls (4.6% vs. 1.2%). SNPHAP analysis revealed this haplotype in all of the 13 patients carrying 9Q and in 3 spouse controls out of 5 carrying 9Q (χ^2=4.93, 1df, nominal p=0.026), and further analysis including the three 9Q associated haplotypes (13 patients and 5 controls) gave values of χ^2=2.49, 1df, p=0.11. Six PD patients carried two combinations of polγ-α changes (6Q+E1143G, 9Q+9Q, 9Q+12Q, 12Q+R722H, R722H+Q1236H, Y831C+Q1236H), and none of the 127 spouse controls had such combinations (Figure 12).

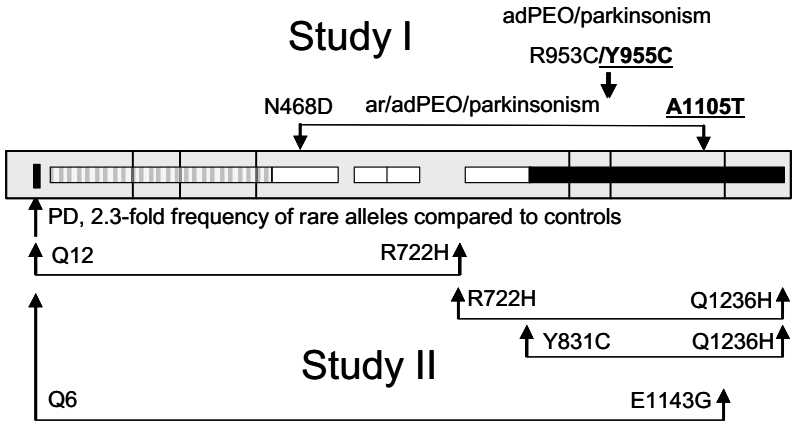

***Figure 12**. **Summary of genetic findings in studies I and II.** Based on study I, Y955C and A1105T (bold) substitutions are associated with parkinsonism. Study II shows association of rare polyglutamine alleles with idiopathic sporadic Parkinson's disease. In addition, different combinations of rare polyγ-α variants were found in the Parkinson's disease patients, but none in the spouse controls.*

2.3 PolyQ variants and age of PD onset (unpublished data)

We analysed the effect of polyglutamine tract length on the age of onset in Parkinson's disease patients. These results suggest that the rare alleles may affect the average age of disease onset. Carriers of the short alleles 8Q and 9Q had later onset than those carrying the long alleles 12Q and 13Q (Figure 13).

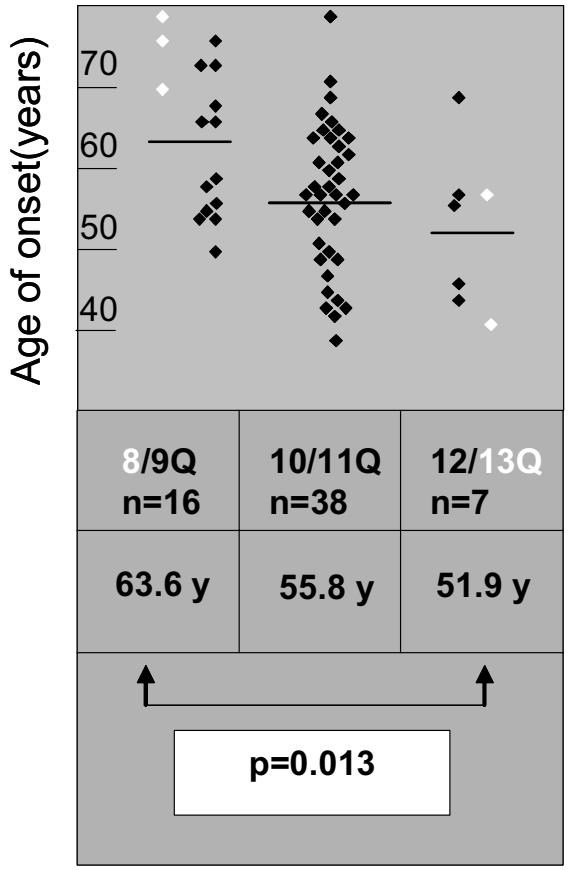

Figure 13. Age of onset in idiopathic sporadic Parkinson's disease patients. *Polyglutamine alleles were grouped in three classes and association of average age-of-onset with polyQ length was analysed by Student's t-test, the p-value given is two-tailed. Rare polyglutamine length variants have correlation with the age of disease onset.*

3. *POLG1* spacer mutations associated with ataxia (III, IV)

3.1 *POLG1* mutations in study III

In all patients we found recessive missense mutations in *POLG1* (III). Seven patients (six familial, one isolated) carried homozygous mutations, three for the previously reported A467T and four for W748S. One isolated British patient was compound heterozygous (A467T/W748S). A467T was a known mutation,[138] whereas W748S was a novel pathogenic mutation *in cis* with E1143G. E1143G alone is a previously reported common polymorphism. W748S and E1143G always occurred together (four patients from two unrelated Finnish families, Figure 14).

3.2 *POLG1* mutations in study IV

We continued our studies with an Austrian family with various neurological symptoms in three generations (IV). *POLG1* sequence analysis showed three amino acid changes: A467T and R627Q *in cis* with a polymorphic variant Q1236H in the index patient. Two of these substitutions affected the spacer region (Figure 14). The phenotype resulting from the heterozygous A467T mutation was late-onset ptosis without ophthalmoplegia. Patients with R627Q/Q1236H had early-onset ptosis and gait disturbance.

The index patient, who was the most severely affected, carried all three changes A467T *in trans* with R627Q/Q1236H. She displayed several CNS manifestations, namely, ataxia, dysarthria, cognitive decline and nystagmus. She also had early-onset PEO, sensory neuropathy and symptomatic restless legs syndrome, which was responsive to treatment with L-dopa. Only sparse ragged-red fibers were seen (<1%) in her muscle biopsy, and Southern blot analysis showed normal-sized mtDNA. Amplification by long-PCR revealed the existence of multiple deleted mtDNA molecules, which clearly exceeded the levels seen in age-matched controls.

To study putative digenic effects in patients with late-onset ptosis heterozygous for A467T,[199] we sequenced the genes encoding ANT1, Twinkle-helicase and 22 mtDNA encoding tRNAs, but did not identify additional mutations. Furthermore, we excluded uneven expression of the two *POLG1* alleles by

showing that the two allelic transcripts were present in equal proportions in the patients lymphoblasts.

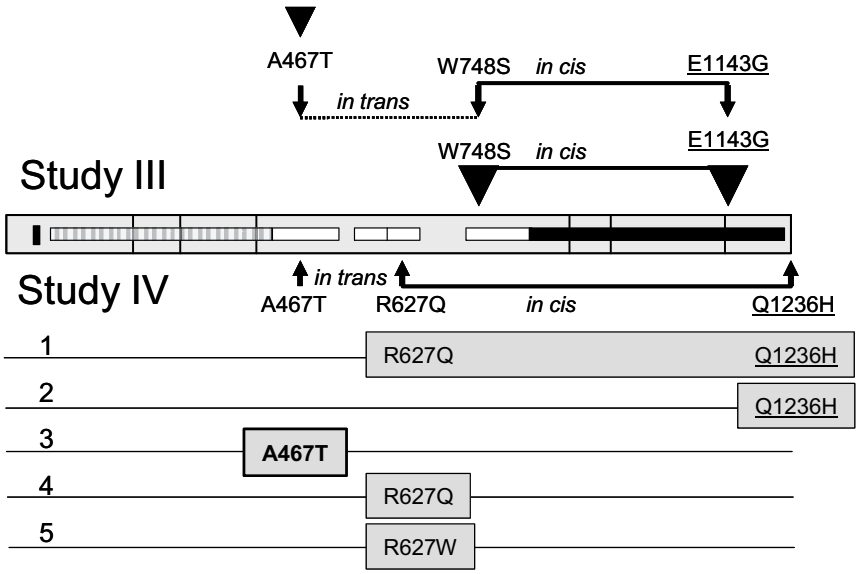

***Figure 14*. Summary of findings in studies III and IV.** *Study III identified new mutations assoiated with ataxia. Study IV identified new mutation associated with ataxia+neuropathy+PEO. The common denominators in these studies were substitutions in spacer region and additional "polymorphic" substitutions (underlined) in the polymerase domain, except in patients in study III who had homozygous A467T substitutions. In both studies the major manifestation was ataxia and neuropathy. Grey boxes below indicate the five polγ-α variants used in biochemical in vitro studies. The A467T substitution clearly impaired DNA polymerase gamma processivity, which was markedly improved by accessory subunit polγ-β. Large black arrowheads indicate homozygous state.*

4. Functional consequences of spacer mutations (IV)

In study IV we analysed for the first time effects of spacer region substitutions A467T, R627Q and the previously reported R627W[132] on DNA and accessory subunit binding and polymerase activity and processivity. In addition, the effect of polymorphic substitution Q1236H was analysed alone or in combination with R627Q to detect a possible accumulative or modifying effect. In addition to mutants described in the Austrian family, the R627W mutation was chosen because it was shown to exhibit a recessive mode of inheritance whereas R627Q/Q1236H mutant allele showed dominant inheritance. The idea was to study biochemically a possible effect of Q1236H on causing R627Q to act as a dominant mutation (Figure 14).

Polγ-α R627Q and R627W variants exhibited specific activities within the normal range, but their DNA-binding affinities were modestly increased, as was also processivity in the case of R627Q. Both DNA binding and processivity were reduced in the A467T variant, and we therefore tested the effect of the polγ-ß subunit on this mutant. The polymerase activity of A467T was <20% of that of the wild-type catalytic core, but, interestingly, accessory subunit polγ-β stimulated this mutant ~20-fold, which was three to four times higher than the effect of the accessory subunit on wildtype polγ-α. Increased stimulation by polγ-β suggests that its functional or physical interactions with polγ-α may compensate partially for the biochemical defect of the A467T mutant *in vitro*.

We also compared the biochemical characteristics of R627Q and Q1236H in combination and separately. When compared with the single R627Q mutant, the double-mutant R627Q/Q1236H enzyme showed ~2-fold higher specific activity in the gapped DNA assay and 1.6-fold higher activity in the ssDNA assay. In the ssDNA assay, the accessory subunit stimulated both mutants similarly, such that the difference in their specific activities remained 1.6-fold. Both processivity and DNA binding were closer to the wild-type values in the double mutant than in R627Q alone.

Discussion

POLG1 mutations have been found to be the most frequent cause of the autosomal dominant and recessive forms of PEO. The results presented in this thesis show that *POLG1* spacer and polymerase region mutations underlie new common clinical phenotypes such as adult-onset recessive ataxia, parkinsonism and premature menopause with recessive or dominant mode of inheritance (I, III, IV, Figure 15). In addition, the preliminary results imply that the rare *POLG1* cag-repeat alleles might associate with idiopathic Parkinson's disease (II).

We have shown that dominant and recessive mutations of *POLG1* may lead to PEO co-segregating with parkinsonism and premature menopause (I). Thereafter, this finding has been confirmed by others. [153,200] PEO represents only one clinical phenotypic expression of *POLG1* mutations. The large differences particularly in phenotypic expressions of recessive *POLG1* mutations are puzzling. Although PEO and sensory ataxia are common consequences of *POLG1* mutations, no single clinical feature is shared by all the patients.[201] Speculations could be made about molecular causes of the observed clinical heterogeneity. Data on familial patients suggest that clinical variability is more marked between families than within families and the contribution of the genetic background and its interaction with the environment may explain the observed clinical heterogeneity. Interestingly, Redon et al. (2006) reported that 12 % of the human genome involves copy number variants, which simply means that individuals do not necessarily have one copy of a genomic region from their mother and one from their father.[202] Such variations are likely to have an impact on disease manifestations and penetrance differences.

One of the major challenges for genetic counselling is the observation that some mutations predicting specific amino acid substitutions can be either dominant or recessive. Thus far there are at least three mutations which have been shown to exhibit both dominant and recessive forms of inheritance: mutations predicting A467T (III, IV), R627Q (IV and [134,158]) and A1105T (I). Not only for counselling but also for basic research it will be important and challenging to find out what is the mechanism underlying this phenomenon. Actually, sequencing can not detect all deletions, splice-site, promoter or intronic mutants, and A1105T, for example, could hence be a recessive mutation combined with an undetectable change. One possibility is that there is a mutation in another PEO associated gene, i.e. a digenic inheritance of the disorder. In addition to sequencing, messenger RNA (cDNA) of the *POLG1* gene should be analysed, both length to detect abnormal splicing and possible expression bias between alleles. Large scale gene deletions should be analysed by Southern blotting. Furthermore, the reported copy number variation[202] should be analysed by quantitative methods such as mini-sequencing.

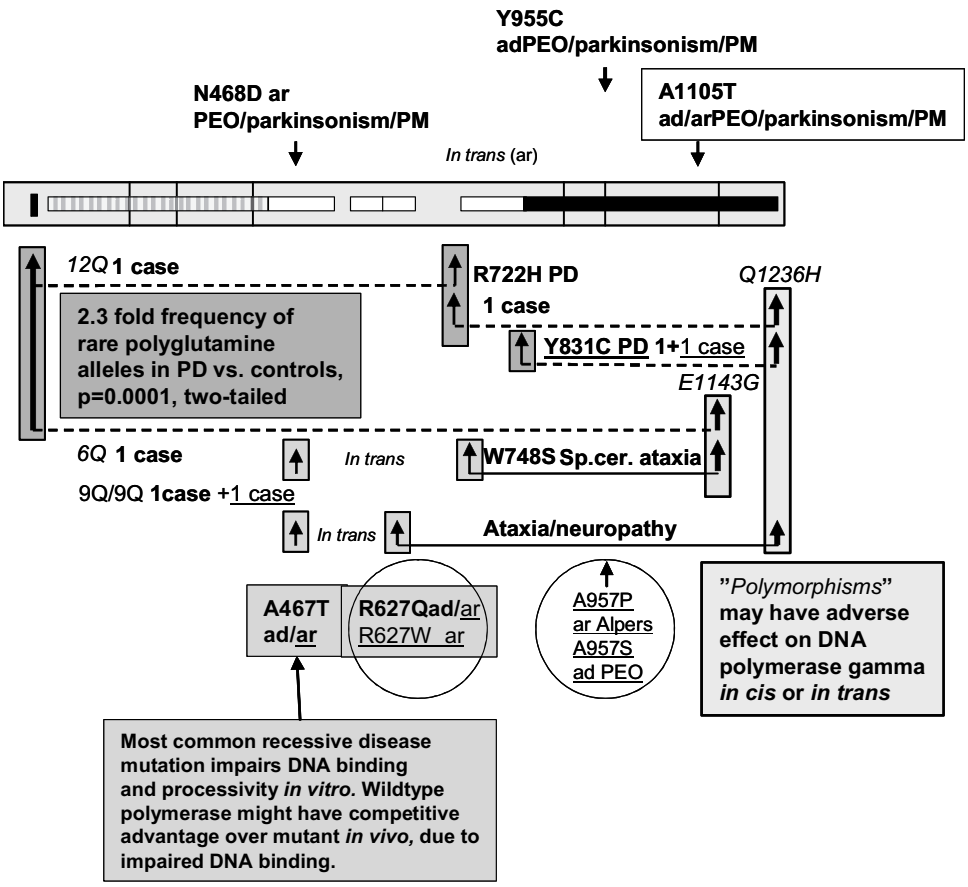

Figure 15. Summary of studies I-IV. POLG1mutations associate with new clinical phenotypes with variable inheritance modes and penetrance differences. *Based on our (**bold**) and previous (underlined) studies by others, it seems obvious that the same amino acid substitution can cause either dominant (ad) or recessive (ar) disease (boxed). In addition, there are also indications that different substitutions at the same position can cause either dominant or recessive disease (circled). According to in vitro studies and genetic findings it seems possible that the "polymorphic" substitutions including rare polyglutamine variants (italics) could have a cumulative pathogenic effect together with another substitution either in cis (solid line) or in trans, dashed lines indicate missing data whether substitutions are in trans* or *in cis. Five Parkinson's disease patients (PD), but none of the spouse controls had these combinations. PM=premature menopause.*

Analysis of the complete *POLG1* gene sequence in 140 Finnish patients with idiopathic sporadic PD showed clustering of rare polyglutamine length variants in the PD subjects, compared to their spouses or healthy population controls and almost 4-fold frequency compared to previously reported Eurasian controls including Finns (II). We also analyzed polγ-α polyglutamine length variation in Finnish patients with other neurodegenerative disorders of putative mitochondrial origin. No clustering was detected in this mixed patient group, either. Overall, this suggests a correlation between *POLG1* encoded polyglutamine variants and Finnish idiopathic PD. If these findings are confirmed in an independent cohort, it could be estimated that approximately 5-10% of Finnish PD patients carry a pathogenic *POLG1* allele. We also showed that the length of rare polyglutamine variants may have correlation with the age of disease onset. This phenomenon is also well known in other neurodegenerative diseases caused by polyglutamine tract expansions, such as Huntington's disease and various spinocerebellar ataxias.[203]

In 536 Finnish chromosomes PD patient and control chromosomes, we detected altogether 17 coding region *POLG1* variants of which five resulted in novel missense changes. These included Q49E in cis with L392V in one PD patient and two controls, suggesting that these two changes arose in one ancestral chromosome. P241L was found in one control, L392V alone in one control and R722H in two patients and one control. The S1230F variant was encountered solely in a PD patient, and it exchanged a phenylalanine for a conserved serine. However, the presence of the same variant in the patient's aged healthy sister suggests that this variant has no role in PD or that it has reduced penetrance. All the novel variants had frequencies of <1% in the control population, but none of them showed a clear association with PD. Furthermore, common amino acid polymorphisms, such as E1143G and Q1236H, showed an even distribution in our PD patients and spouse controls. The many previously unreported changes detected in this study suggest that either *POLG1* is naturally prone to undergo *de novo* mutagenesis, reflects the relatively small number of previously analysed complete *POLG1* gene sequences, or shows that specific *POLG1* alleles are enriched in the Finnish population.

Two previous studies have analyzed a partial *POLG1* gene sequence in PD patients. A study of 22 patients did not report a correlation between the polyQ repeat length and PD.[204] However, this series was too small to warrant conclusions on genetic factors underlying a disorder involving several other disease genes. The report did not mention the age of the controls, and the samples were analyzed by polyacrylamide electrophoresis, which cannot detect polyQ-alleles with amino acid substitutions, such as Q43R and Q49E. The other study reported analysis of selected exons 7, 16, 18 and 21 of *POLG1* in 140 unselected PD patients, and also failed to find PD-specific changes, although they found two similar cases among 140 PD patients as we did (Y831C+Q1236H and

9Q/9Q), these were seen neither in our nor their controls. [171] Part of the sequence analysis was done by dHPLC and the polyglutamine encoding region was analyzed by capillary gel electrophoresis. Based on our own experience and published reports, dHPLC may fail to detect many of the *POLG1* sequence changes.[128] These reports could not exclude the role of *POLG1* in idiopathic PD.

Most cases of ataxia and *POLG1* spacer mutations presented here (III,IV) lacked all major signs of mitochondrial myopathies, such as ragged-red fibres or mtDNA deletions in Southern blot or long-PCR analysis, leaving the mitochondrial etiology of their disease undetected in routine histological and DNA analyses of muscles.[149,205,206] Since the accumulation of mtDNA deletions in tissues putatively causes pathology,[207] the absence of muscle mtDNA deletions in Southern blot analysis correlates with the absence of clinical and morphologic abnormalities in the muscle characteristic of PEO.

Up to date there are more than 30 confirmed homozygous W748S+E1143G patients in Finland and quite likely several homozygous carriers who have not been affected yet because of their young age. The W748S+E1143G associated disorder was recently named as MIRAS (Mitochondrial Recessive Ataxia Syndrome).[111,208] Although the patients have the same homozygous mutation, the clinical phenotypes are heterogenous with a characteristic set of features, including ataxia, peripheral neuropathy, dysarthria, mild cognitive impairment, involuntary movements, psychiatric symptoms, and epileptic seizures. According to a recent report,[209] the incidence of MIRAS is approximately 1 in 80 000, which means an allele frequency of 0.35%. Consequently there are over 40 000 heterozygous carriers (1 in 125) and approximately 60 homozygous carriers in Finland. There is also evidence suggesting that one heterozygous carrier might also be affected mildly.[172] The W748S+E1143G combination has also been found in New Zealand, United States, Australia and Europe, and haplotyping has revealed that they all share a single common ancient European ancestry.[209]

In ataxia, amino acid substitutions A467T, W748S, E1143G, Q1236H and R627Q alter evolutionarily conserved amino acid residues (III, IV). The contribution of E1143G to the phenotype remains unclear, although preliminary *in vitro* functional assays show some altered biochemical characteristics,[210] (IV). Because E1143G is present *in cis* with W748S, it cannot be concluded that W748S or E1143G alone is sufficient to cause disease in homozygous or compound heterozygous patients. This situation is similar to the *POLG1* mutations T251I *in cis* with P587L in arPEO patients of different ethnic origins.[149,155] Therefore, segregation analysis of novel low-frequency mutations remains crucial to the determination of their pathogenic nature. *In vivo* functional studies on DNA polymerase gamma are still unavailable and *in vitro* studies may not detect all known and potential unknown functional defects, since functional studies include

only a restricted set of experiments including DNA and accessory subunit binding and polymerase activity and processivity studies.

All functional *in vitro* studies reported previously have been based solely on analyses of dominant mutations affecting the polymerase domain of polγ-α.[84,108,194] Our studies showed for the first time that the spacer region mutant, A467T, has a clear and severe biochemical phenotype indicating impaired polymerase function. This most likely results from reduced DNA binding and thus affects the initiation of DNA strand elongation and attachment on the template during elongation. Part of the pathogenic mechanism may be explained by impaired interaction with the accessory subunit polγ-β. Other mutations did not show equally clear defects in their catalytic or DNA binding properties. Substitutions affecting different regions of polγ-α have different pathogenic mechanisms, which do not always directly affect the exonuclease or polymerase functions of the enzyme.

We showed *in vitro* that a common polymorphism may have a modulating effect on the biochemical characteristics of the polγ-α protein when occurring alone or in combination with a pathogenic mutation (IV). Double mutant R627Q/Q1236H had increased polymerase activity compared to R627Q alone. The intriguing possibility remains that these sequence changes could have a synergistic or accumulative effect on polγ-α function and it could be possible that those proteins which have less impaired DNA or accessory subunit binding, act more competitively because of their ability to compete better with wild-type protein (dominant negative effect). A467T shows severely impaired DNA binding and most likely has less competitive ability with wild-type to bind DNA *in vivo* (loss of function effect). It could be possible that in mildly affected patients (A467T) there are other physiological factors that might modify this binding capacity leading to more competitive binding, and hence to mild clinical manifestation.

Conclusions

We show in these studies that *POLG1* mutations are relatively common causes of neurodegenerative disorders. Our data that *POLG1* mutations can also underlie ataxia, parkinsonism/PD and premature menopause are essential for studies of the genetics of these disorders and for clinicians diagnosing neuromuscular diseases. Based on our results (I-IV), a *POLG1* diagnostic routine has been set up in Helsinki University Central Hospital (HUSLAB) and new *POLG1* mutations have been found in patients with neuromuscular disorders.

The position and nature of amino acid changes in polγ-α seem to have a strong but incomplete correlation with the mode of inheritance and the clinical manifestation, so that exo- and spacer mutations are predominantly recessive whereas dominant mutations cluster mostly in the polymerase domain. The mode of inheritance is occasionally ambigious; one mutation seems to have both recessive and dominant modes of inheritance, although the existence of deletions, intronic or promoter region mutations has not been excluded. In addition, a wide spectrum of symptoms and degrees of penetrance can be detected in different members of the same family, and even more evidently between different families and/or sporadic cases. Furthermore, the muscular manifestations characteristic of PEO are absent in some patients, for example in recessive spinocerebellar ataxia syndrome (MIRAS) caused by a homozygous mutation predicting a W748S substitution. On the other hand, the A467T substitution causes a wide variety of severe clinical phenotypes in homozygous individuals between distinct families with or without PEO. This clinical heterogeneity might be due to different sets of polymorphic modifier genes between individuals.

Complete analyses of the *POLG1* gene in 140 Finnish Parkinson's disease patients revealed a clear difference in rare polyglutamine variant frequencies compared to pooled population controls. The question of whether these length variants are pathogenic, risk factors or modifier genes needs to be studied further by larger patient series, familial co-segregation studies and *in vitro* and *in vivo* functional analysis.

Biochemical characterization of pathogenic amino acid substitutions *in vitro* show that the spacer region of polγ-α has a potential role in template DNA binding and could also have uncharacterized enzymatic function or functions not revealed by the methods so far used. Furthermore, the common A467T substitution has a clear defective biochemical phenotype, which is in accordance with the severe clinical manifestations seen in homozygous patients. There is also evidence that amino acid polymorphisms might have a role in *POLG1* associated syndromes. Our functional and genetic studies provide support for this hypothesis.

Future prospects

Mutations in the *POLG1* gene for the catalytic subunit of polG have been linked to several mitochondrial disorders, including progressive external ophthalmoplegia, sensory and ataxic neuropathy, Alpers syndrome and parkinsonism. Up to date, over 80 pathogenic mutations have been identified in the *POLG1* coding region. The identification of *POLG1* as a major disease locus for human mitochondrial disorders has stimulated research on *POLG1* and also highlighted a number of mysteries in the biology of mtDNA and human disease. Since *POLG1* mutations have turned out to be a common source of a wide variety of distinct pathological states, it would be of major interest to study in detail the molecular influence and pathogenic mechanisms on the mtDNA and OXPHOS system both down- and upstream. This research should also address the question of why some post-mitotic tissues are not affected, i.e. what is the mechanism on avoiding defective consequences. Detailed knowledge would be a prerequisite for rational development of conceivable cure and medication for POLG-associated syndromes. Mutations of *POLG1* might provide unique opportunity to develop treatments since, unlike other nuclear OXPHOS defects, there is a possibility to study patient cohorts large enough, with a common denominator, i.e. with the same mutation.

One of the major questions to be studied in the future is the mechanism of mtDNA replication. Most of the essential proteins involved in mtDNA replication have likely been identified, and reconstructed mtDNA replication forks have been studied *in vitro*. This means that extensive functional studies both *in vitro* and *in vivo* need to be conducted. Currently, there are two competing models of mtDNA replication and further biochemical investigation is needed to develop a consistent model. Studies of dysfunctional enzymes resulting from disease mutations should yield new insights into the mechanism of mitochondrial DNA replication. Furthermore, one crucial aspect in these studies would be the role of the structure of substrate; mtDNA has a complex three-dimensional structure which could also have multiple conformations related to gene expression, replication, repair and other possibly metabolic factors.

One interesting and quite recent approach that has been applied successfully is the new animal model, namely the domestic dog (*Canis familiaris*), which is physiologically more similar to human than mouse. Due to inbreeding within pedigrees, dogs have disorders resembling *POLG1*-associated syndromes seen in humans, such as ataxia, tremor, neuro- and myopathies. (See Online Mendelian Inheritance in Animals; http://omia.angis.org.au/). Quite likely they also have mitochondrial disorders and pathogenic mutations in *POLG1* or in other known human disease orthologs, although the manifestations can be quite different from humans. Many breeds are well registered with detailed information of pedigrees, which could help to find either new disease genes or mutations in

genes known to associate with human disorders. Another advantage would be the availability of tissue material in order to accomplish distinct omics- and histological studies and to develop and test treatments.

Exploitation of such animal models has also ethical advantages over transgenic models. For example, even those who oppose transgenic animal models seem to accept this approach, mostly because it is regarded as a sort of “natural” inevitability like human diseases and it might also benefit animals if new treatments could be developed on this basis.

Finally, the analysis of *POLG1* mutations should be developed further, in addition to sequencing of coding regions, also analysis of the allele copy numbers, promoter region defects, intronic mutations and deletions should be considered. Furthermore, also possible digenic mutations in other PEO-genes should be analysed in the case of inheritance mode discrepancy.

Acknowledgements, that is to say :

“So Long, and Thanks for All the Fish”

Douglas Adams: The Hitchhiker's Guide to the Galaxy

Most of all I am grateful to Professor Anu Wartiovaara for this great opportunity she gave me to do this research, which is not only engrossing but also has meaning in everyday life, for patients, science as well as for all-round education.

I am deeply indebted to Professor Anna-Elina Lehesjoki and Associate Professor David Thorburn for reviewing the manuscript. They shared some point of views, but the most fruitful outcome to me was that they also had quite different point of views from their own premises. I think this was essential for the clarity of this thesis.

I am also grateful to Professor Kalervo Hiltunen for his acceptance of the post of the official opponent.

All co-authors deserve great thanks.

The fact is that my head is totally empty at this moment and time is running out. I apologize that I have not mentioned all those people by name to whom I should be greatfull. I will do it personally.

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